Lab Notebook

Gate construct

Gate 1

Step 1

Gate 1- Background:

This gate is the changeable gate. In other words, this gate is used in order to control the system to detect the desirable substance.

The system we have designed can detect any substances, but first needs to be adjusted for a specified material.

Gate 1 comprised with the following parts:

- A promoter that detects the analyte, for demonstration we used p_tet that detect the antibiotics tetracycline.
- luxI- an enzyme that produces AHL, a quorum sensing molecule. The production of AHL is crucial for the signal enhancement.
- Double terminator.

While combined with the other 2 gates the system will detect any desirable substance in a very low concentration.

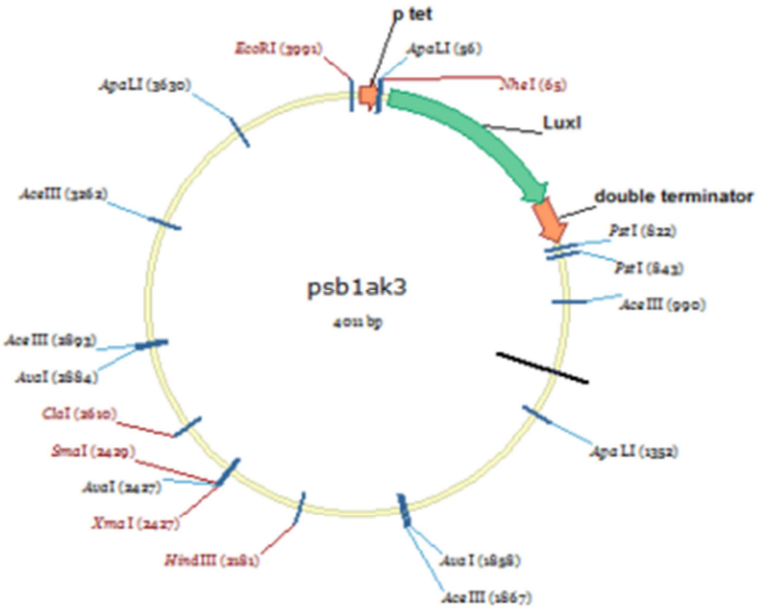
Our work on creating this gate:

- At first we used the pSB1AK3 and by reverse PCR we cloned the p_tet promoter into the plasmid.

- Second, we did Gibson's assembly protocol to insert the luxI gene followed the p_tet.

* The pSB1AK3 was already consisted with the double terminator.

Following is the full plasmid contains Gate 1:



Reverse PCR and sequencing

Reverse PCR- First attempt

29/12/13

The purpose of the reaction was to add the p_tet promoter to the psb1AK3 plasmid that is already containing the double terminator.

The reaction was with the primers:

number	primer		uniqe	length	Tm	hair pin	self	hetero	% GC	Tm	% GC	name
1	tcagtgatagagatactgagcacgtgcacaacgctagcccaggcatcaaataaaac	forward	+	56	69.3	-5.97	-16.48	-20.25	46.4	46.2	38.9	F revers
2	tagggatgtcaatctctatcactgatagggactctagaagcggccgcga	reversed	+	49	69	-3.11	-22.78	-20.25	51	60	66.7	R revers

The expected band is in size of 3429 bp (the whole plasmid without the luxI: 4008- 579=3429 3429 bp)

2 stage PCR (55◦C, 69◦C)
Reaction mix:

Competent	Volume (µl)
Phusion reaction buffer (X10)	10
dNTP's (10 mM)	1
P_tet Forward	2.5
P_tet Reverse	2.5
pSB1AK3:ter (144.5 ng/µl)	5
Phusion hot start II	0.5
DMSO	1.5
UPW	27
Total	50

PCR program - 2 stage PCR (55◦C, 69◦C)

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
5 cycles	98 55 72	10 sec 30 sec 1 min
27 cycles	98 69 72	10 sec 30 sec 1 min
Final extension	72	10 min
Hold	4	

Results : No bands.

30/12/13

Gradient PCR (55-70 ◦C, 6 PCR tubes)

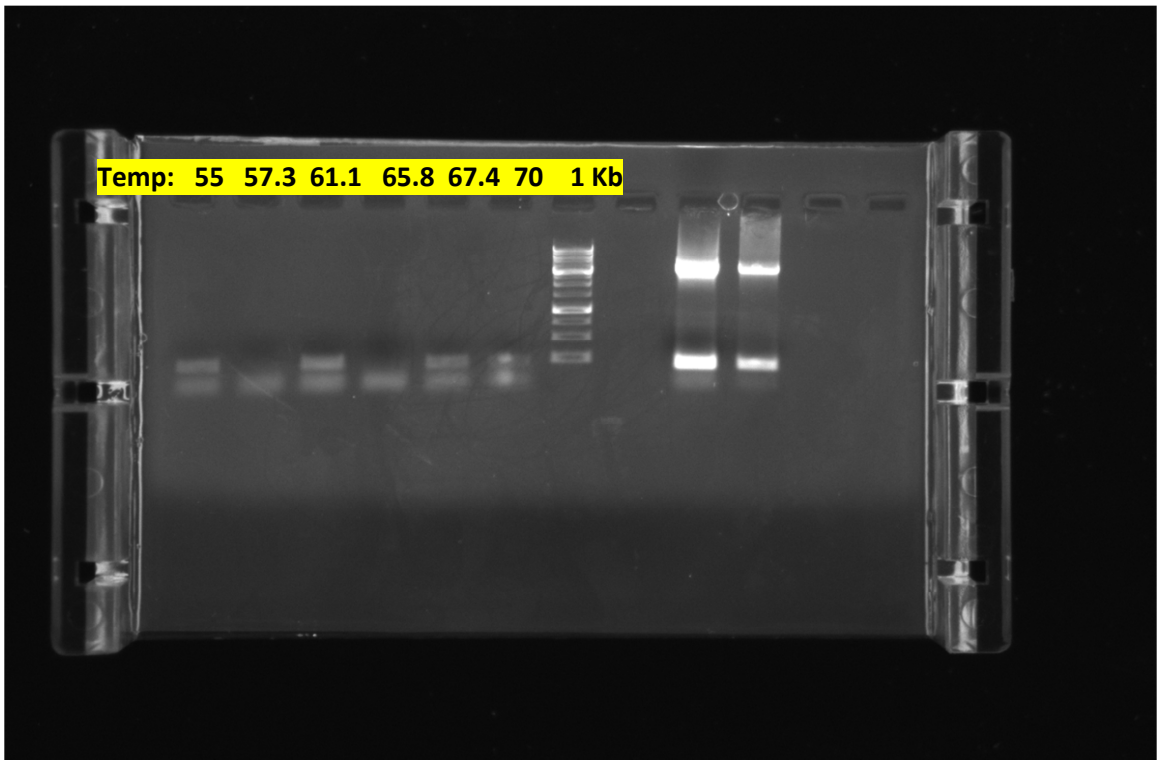
Reaction mix:

Competent	Volume (µl)
Phusion reaction buffer (X10)	10
dNTP's (10 mM)	1
P_tet Forward	2.5
P_tet Reverse	2.5
pSB1AK3:ter (144.5 ng/µl)	5
Phusion hot start II	0.5
DMSO	1.5
UPW	27
Total	50

PCR program - gradient PCR (55◦C - 70◦C)

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
35 cycles	98 55, 57.3, 61.1, 65.8, 67.4, 70 72	10 sec 30 sec 1 min
Final extension	72	10 min
Hold	4	

Results : No bands.



05/01/14

Gradient PCR (69, 71 ◦C, 2 PCR tubes)

Reaction mix:

Competent	Volume (µl)
Phusion reaction buffer (X10)	5
dNTP's (10 mM)	0.5
P_tet Forward	1.25
P_tet Reverse	1.25
pSB1AK3:ter (144.5 ng/µl)	2.5
Phusion hot start II	0.25
DMSO	0.75
UPW	13.5
Total	25

PCR program - gradient PCR (69◦C , 71◦C)

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
35 cycles	98 69, 71 72	10 sec 30 sec 1 min
Final extension	72	10 min
Hold	4	

Results : No bands.

Gradient PCR (55-69 ◦C,7 PCR tubes: 2 of them is with Q5 enzyme instead of Phusion hot start II, 65 and 69 ◦C)

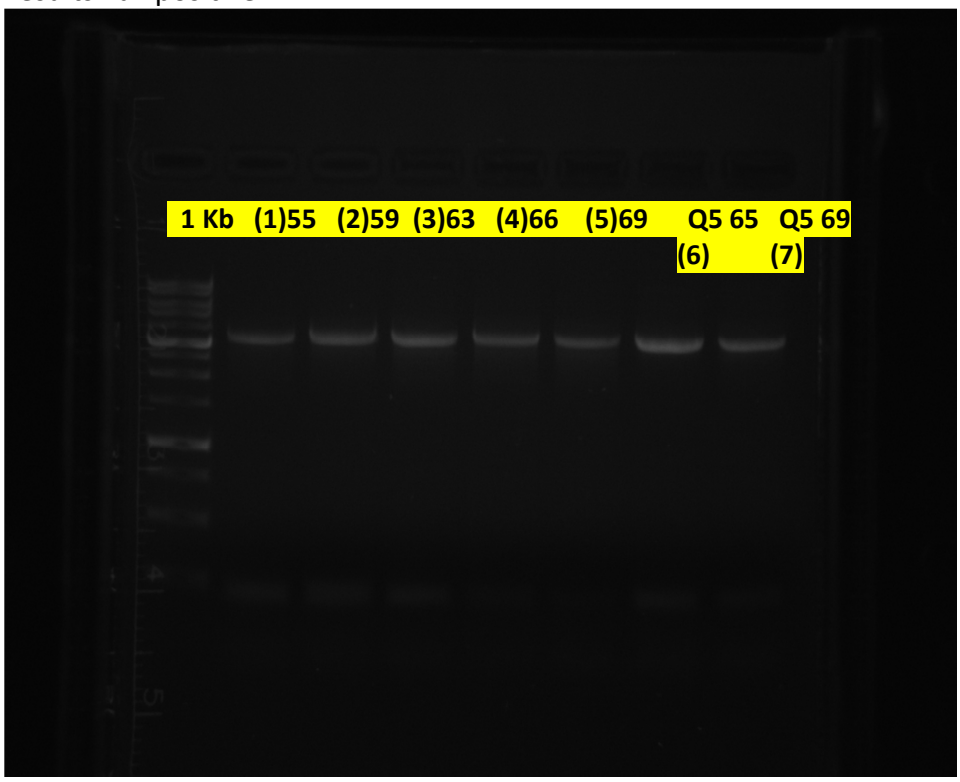
Reaction mix:

Competent	Volume (µl)
Phusion reaction buffer (X10)	5
dNTP's (10 mM)	0.5
P_tet Forward	1.25
P_tet Reverse	1.25
pSB1AK3:ter (144.5 ng/µl)	2.5
Phusion hot start II	0.25
DMSO	0.75
UPW	13.5
Total	25

PCR program - gradient PCR (69◦C , 71◦C)

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
35 cycles	98 55, 59, 63, 66, 69 + Q5: 65, 69 72	10 sec 30 sec 1 min
Final extension	72	10 min
Hold	4	

Results : all positive



06/01/14

Blunt ligation over night

12/01/14

Heat shock Transformation

13/01/14

Colony PCR
1-5 colonies were picked

Starter was made from colonies 2 + 3

14/01/14

Glycerol stock: colony 2- F1, colony 3-F2
Mini-prep: colony 2: 222.0 ng/µl
colony 3: 263.5 ng/µl
Sent to sequencing with the following primers:

Primer Name	**Sequence 5' to 3'**
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG

17/01/14

Sequencing results: 3 bp were missing-> new primers with c/g at the edges.

```
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GAGGTATGTNNCNGNGCTNCAGANTTCTTGAAGTGGTGGCCTANCTNNNNTANNCNANAANANAGTNTTTNNTATCTGCN
CTNTGNTGAANCCAGTNACCTTNNNAAAANNNTTGGNNGCTCNNGNNNNNNNNNNAACNANCGCNNNNNCGNNNNNTTT
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AAAAGGCCGCGTTGCTGGcGTTTTTCCNNAGGCTCNGNCCCNNTGACGAGCATCACAAAAATCNNNGCTCAAGTCAGAGG
     TGGCGAANCCNGACAGGACTATNNNNATACNANGCGTTTNNCNCTGGAAGCTCCNTCGTGCNGCTCTCCTGTTNN
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Reverse PCR, Phosphorylation and Blunt ligation and transformation

Reverse PCR- Second attempt

24/02/14

The reaction was with the new primers:

number	primer		uniqe	length	Tm	hair pin	self	hetero	% GC	Tm	% GC	name	Anneal
1	**Cta**tcagtgatagagatactg Agcacgtgcacaacgctagc ccaggcatcaaataaaac**g**	forward	+	60	69.1	-6.23	-16.48	-24.29	46.7	50	66.6	F revers	72
2	Ggatgtcaatctctatcactgatagg gactctagaagcggccgcga**attc**	reversed	+	50	68.3	-3.73	-22.78		50	57.8	42.9	R revers	

Reaction mix:

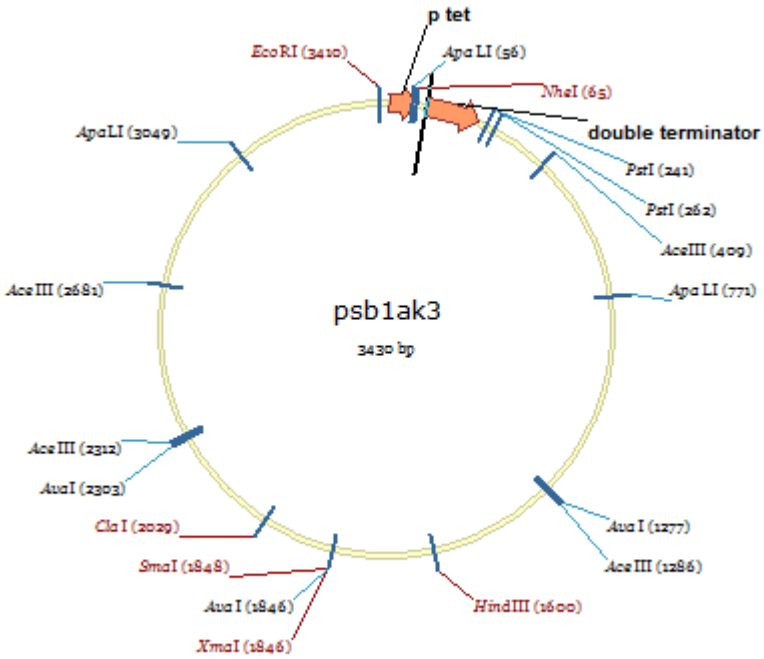
Competent	Volume (µl)
Phusion reaction buffer (X10)	5
dNTP's (10 mM)	0.5
P_tet Forward	1.25
P_tet Reverse	1.25
pSB1AK3:ter (144.5 ng/µl)	2.5
Phusion hot start II	0.25
DMSO	0.75
UPW	13.5
Total	25

PCR program - gradient PCR (69◦C , 71◦C)

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
35 cycles	98 69 72	10 sec 30 sec 1 min
Final extension	72	10 min
Hold	4	

Total reaction: 3 test tubes- each tube 25 microliter.
The purpose of the reaction was to add the p_tet promoter to the psb1AK3 plasmid that is already containing the double terminator.

The expected band is in size of 3430 bp.
The following map:

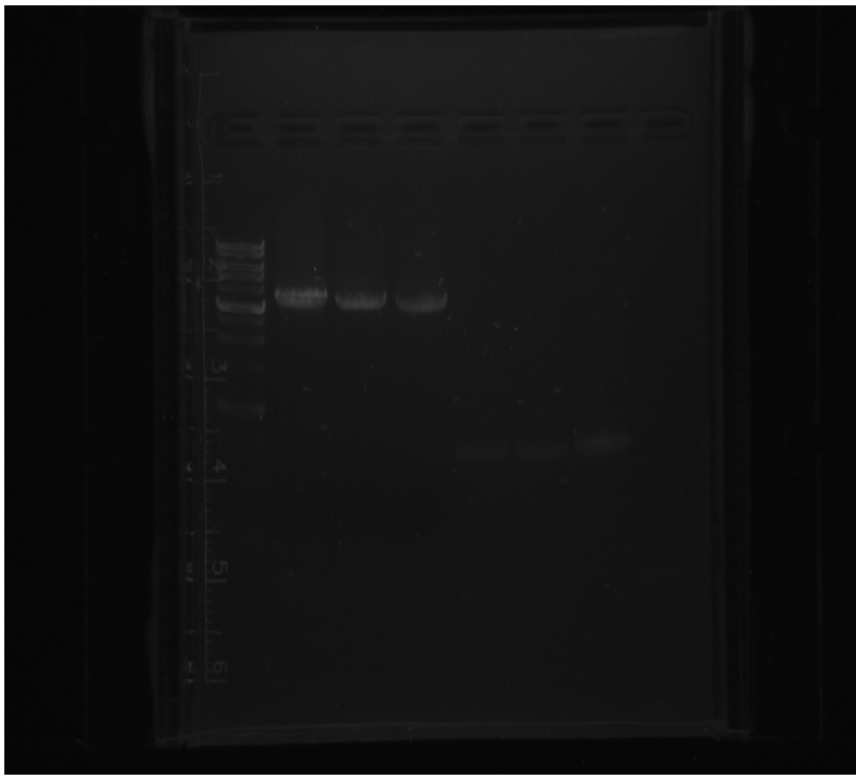


Results for reverse PCR experiment (Gel 1- with 1000 bp ladder)

26/2/14

Results for reverse PCR experiment (Gel 2- with 1kb ladder)
From left to right

1. 1kb ladder
2. 1 test tube
3. 2 test tube
4. 3 test tube



The expected band was received between 3000 to 4000 bp.

26/02/14

Clean products with the PCR clean kit

All 3 test tubes combined - 40 microliter

Concentration: 69 ng/microliter

Phosphorylation and Blunt ligation

O/N at 16 degrees

Transformation to top 10: 3 attempts

02/03/14

Transformation to new top 10
Results: No growth

04/03/14

Transformation to old top 10 (heat shock)
Results: No growth

05/03/14

Transformation to old top 10 (heat shock)
Results: No growth

We will repeat the Phosphorylation and Blunt ligation.

Phosphorylation and Blunt ligation - trial 2

06/03/14

With enzymes from Roee's lab
1 hour (not 30 min) for phosphorylation

Transformation to top 10

07/03/14

Transformation to new top 10
Results: growth

Colony PCR

11/03/14

8 colonies were picked

The F primer is 23bp long and 117 bp before the p_tet
The R primer is 23bp long and 178 bp after the terminator

Primers info:

Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

Reaction tube (total volume= 20µl):

Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony
UPW	6

PCR program:

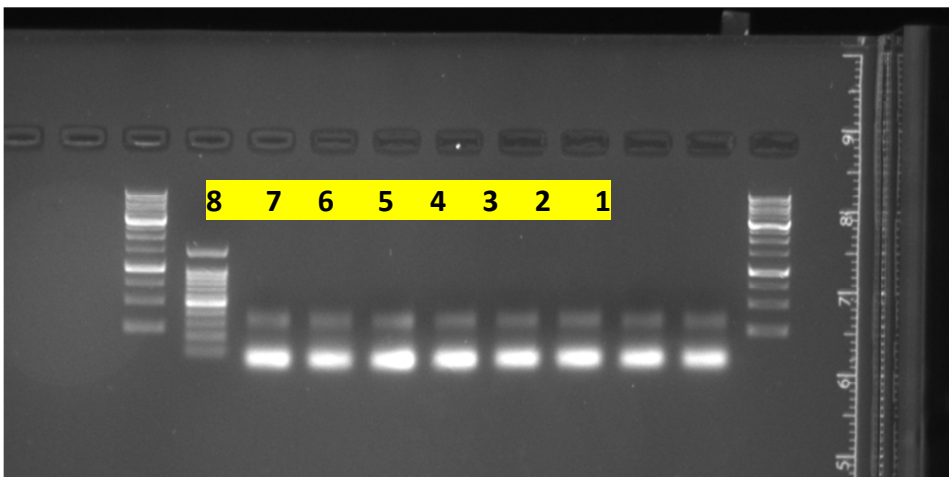
Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	30sec
Final extension	72°C	10min
Hold	4°C	-

Total bp added: 341bp

\First colony PCR with Gibson primers:

primer		uniqe	length	Tm	hair pin	self	hetero	% GC	Tm	% GC	name
gtttaaaaaagcagtcttaaattagccaggcatcaaataaaacg	forward	+	40	59.2	-0.15	-8.74	-10.68	30	50.5	42.1	F gibson
cgatttttttatcattatagtcatgggggtttctcctctttaatgctagcgttgtgcacgtgc	reversed	+	64	68.5	-2.89	-16.48		42.2	72	70	R gibson

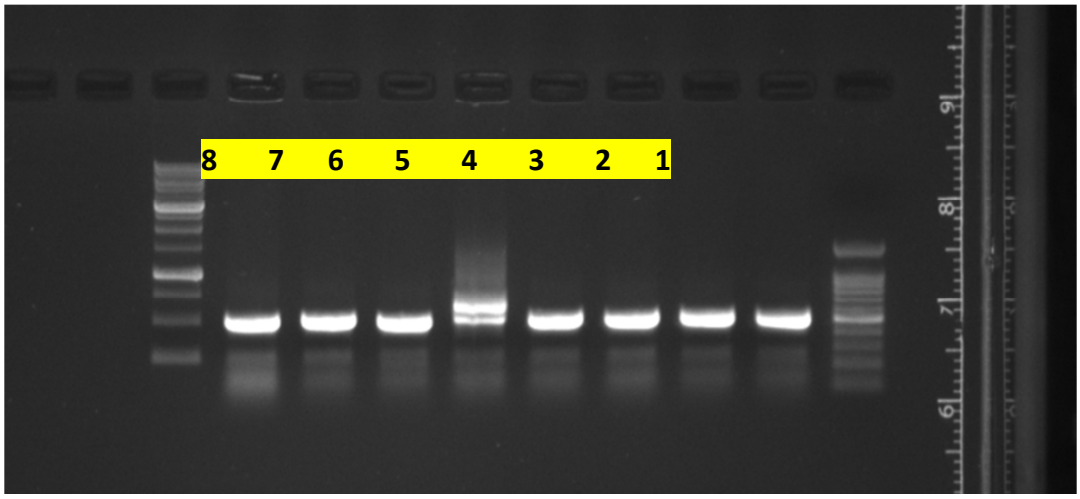
Result: no positive result



12/03/14

Second colony PCR with sequencing primers (pSB1C3_Ver_S and pSB1C3_Ver_AS)- excepted band of 537 bp

All colonies except of colony 5 were positive.



MiniPrep+Sequencing results

12/03/14

Sequencing of Colony 4. -80 freezer: D9
results: 3 bp were missing between the two primers (each primer contain half of the promoter).

```
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Sequencing of Colony 1,2 and 7. -80 freezer: E2, E3, E4 respectively.
results:
p_tet was **successfully** added to colony 2

Primers for sequencing:

Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

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GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGA
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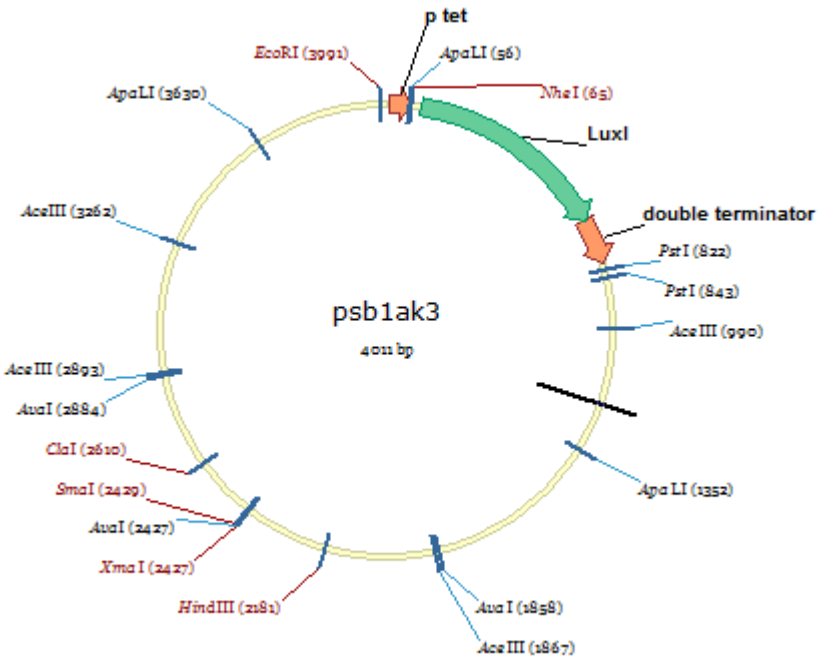
Step 2



Before Gibson 1

20/03/14

Experiment: amplification of the plasmid and the gene luxI before Gibson assembly.

luxI:

Primers info:

primer		uniqe	length	Tm	hair pin	self dimer	hetero dimer	% GC	Tm relavent	% GC relevant	name
atgactataatgataaaaaaatcggatt	forward	+	25	47.8	0.52	-3.61	-10.68	24	50.8	21.4	F gibson luxI
ctaatttaagactgctttttttaaactg	reversed	+	24	48.4	-0.33	-8.74		25	50.6	25.9	R gibson luxI

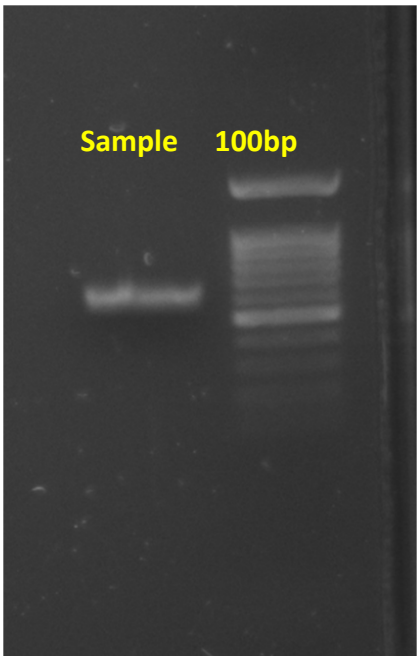
Reaction tube:

µl	Component
31	upw
10	5xPhusion buffer
1	dNTP's
2.5	F primer
2.5	R primer
1	Gene (9.77 ng/µl)
1.5	DMSO
0.5	Phusion DNA polymerase
50	Total

PCR program:

Stage	Temp.	time
Initial denaturation	98°C	
35 cycles	98°C	30 sec
	50°C	30 sec
	72°C	50 sec
Final extension	72°C	10min
Hold	4°C	-

Before Passover - luxI (582 bp) was successfully amplified (2/4/14) - 333.5 ng/µl



Plasmid:

Primers info:

primer		uniqe	length	Tm	hair pin	self	hetero	% GC	Tm	% GC	name
gtttaaaaaagcagtcttaaattagccaggcatcaaataaaacg	forward	+	40	59.2	-0.15	-8.74	-10.68	30	50.5	42.1	F gibson
cgatttttttatcattatagtcatgggggtttctcctctttaatgctagcgttgtgcacgtgc	reversed	+	64	68.5	-2.89	-16.48		42.2	72	70	R gibson

Reaction tube:

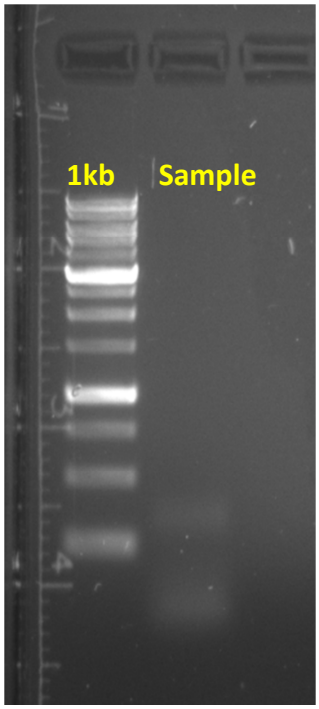
µl	Component
31	upw
10	5xPhusion buffer
1	dNTP's
2.5	F primer
2.5	R primer
1	Plasmid (8.65 ng/µl)
1.5	DMSO
0.5	Phusion DNA polymerase
50	Total

PCR program:

Stage	Temp.	time
Initial denaturation	98°C	
35 cycles	98°C	30sec
	60°C	30sec
	72°C	2 min
Final extension	72°C	10min
Hold	4°C	-

The plasmid (about 4100 bp) with the overlapping ends- failed (177.5 ng/µl) .

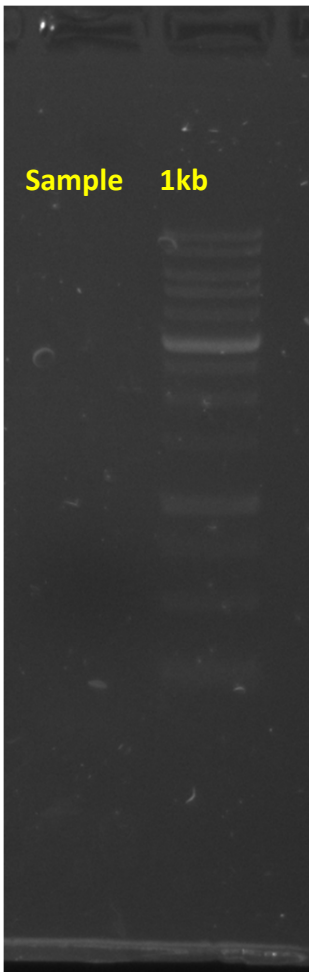
23/03/14



Gradient PCR program: 55, 58, 63, 68 C^0- field (no picture).

Stage	Temp.	time
Initial denaturation	98°C	
35 cycles	98°C	30sec
	60°C	30sec
	72°C	2 min
Final extension	72°C	10min
Hold	4°C	-

24/03/14



Result: no positive band

Before Gibson 2

On Passover new primers were designed and ordered : before that the overlapping was on the plasmid's primers, and now- the overlapping is on the gene's (luxI) primers. It means that the overlap was added to the gene instead of adding it to the plasmid.

29/03/14

Amplification of the plasmid (4011 bp)- succeed (307 ng/µl):

Plasmid:

Primers info:

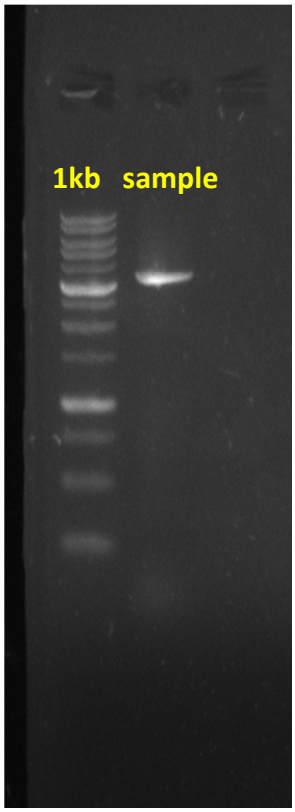
primer		uniqe	length	Tm	hair pin	self dimer	hetero dimer	% GC	Tm relavent	% GC relevan	name
ccaggcatcaaataaaacgaaag	forward	+	23	52.9	1.75	-3.61	-6.9	31.1	-	-	F plasmid
gctagcgttgtgcacgtgc	reversed	+	19	60.1	-1.79	-16.48		63.2	-	-	R plasmid

Reaction tube:

µl	Component
31	upw
10	5xPhusion buffer
1	dNTP's
2.5	F primer
2.5	R primer
1	Plasmid (8.65 ng/µl)
1.5	DMSO
0.5	Phusion DNA polymerase
50	Total

PCR program:

Stage	Temp.	time
Initial denaturation	98°C	
35 cycles	98°C	30sec
	60°C	30sec
	72°C	2 min
Final extension	72°C	10min
Hold	4°C	-



30/03/14

Amplification (2 stage) of the luxI + overlapping ends (660bp)- **succeed** (334 ng/µl) :

luxI:

Primers info:

primer		uniqe	length	Tm	hair pin	self	hetero	% GC	Tm	% GC	name
gcacgtgcacaacgctagcattaaagaggagaaaccccccatgactataatgataaaaaatcg	forward	+	64	68.2	-2.72	-16.48	-10.68	42.2	45.5	20.8	F gibson
gtctttcgactgagcctttcgttttatttgatgcctggctaatttaagactgctttttaaac	reversed	+	63	66.5	-2.81	-8.74		36.5	47.5	24	R gibson

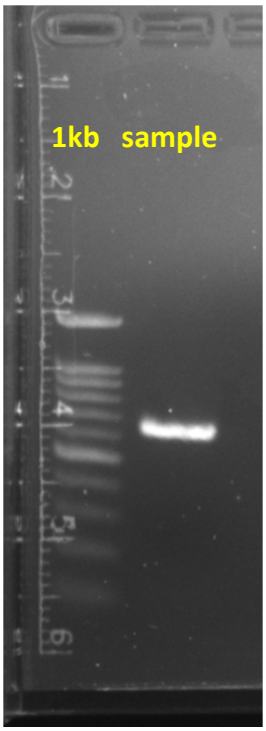
Reaction tube:

µl	Component
31	Upw
10	5xPhusion buffer
1	dNTP's
2.5	F primer
2.5	R primer
1	Gene (9.77 ng/µl)
1.5	DMSO
0.5	Phusion DNA polymerase
50	Total

2 stage PCR program:

stage	Temp C^0	time
Initial denaturation	98	30 sec
5 cycles	98	10 sec
	45	30 sec
	72	1 min
30	98	10 sec
	65	30 sec

	72	30 sec
Final extension	72	10 min
Hold	4	



Gibson assembly

01/05/14

The luxI and the plasmid product were diluted fold 10.

Calculations:

General: $DNA\ concentration\ \left[\frac{pmol}{\mu l}\right] = \frac{DNA\ concentration\ \left[\frac{ng}{\mu l}\right] \times 1000}{length\ [bp] \times 650}$

F overlap: 64-28=36 36 bp
R overlap: 63-27=36 36 bp
luxI= 582 bp

Total: 36+36+582=654 660 bp

$$DNA\ concentration\ (luxI)\left[\frac{pmol}{\mu l}\right] = \frac{33.4\left[\frac{ng}{\mu l}\right] \times 1000}{660[bp] \times 650} = 0.078$$

$$DNA\ concentration\ (luxI)\left[\frac{pmol}{\mu l}\right] \times V[\mu l] = 0.15\ [pmol]$$

$$0.078\left[\frac{pmol}{\mu l}\right] \times V[\mu l] = 0.15\ [pmol]$$

$V = 1.9\ [\mu l]$

The completed plasmid is 4011 bp, so plasmid with no luxI= 4011-582=3429bp

$$DNA\ concentration\ (plasmid)\left[\frac{pmol}{\mu l}\right] = \frac{30.7\left[\frac{ng}{\mu l}\right]\times 1000}{3429\,[bp]\times 650} = 0.014$$

$$DNA\ concentration\ (plasmid)\left[\frac{pmol}{\mu l}\right] \times V[\mu l] = 0.05[pmol]$$

$$0.014\left[\frac{pmol}{\mu l}\right] \times V[\mu l] = 0.05[pmol]$$

$$V = 3.6\ [\mu l]$$

Component	V [µl]
Total Amount of fragments	3.6+1.9=5.5
gibson Assembly Master Mix (2X)	15
Deionized H2	4.5
Total Volume	25

Transformation

We preformed a transformation of the Gibson Assembly product to TOP10 bacteria.
The bacteria were plated on Amp plates (100 µl).

02/05/14

After an overnight incubation colonies have grown on the plate.

Colony PCR

04/05/14

8 colonies were picked

The F primer is 23bp long and 117 bp before the p_tet
The R primer is 23bp long and 178 bp after the terminator
Primers info:

Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

Reaction tube (total volume= 20µl):

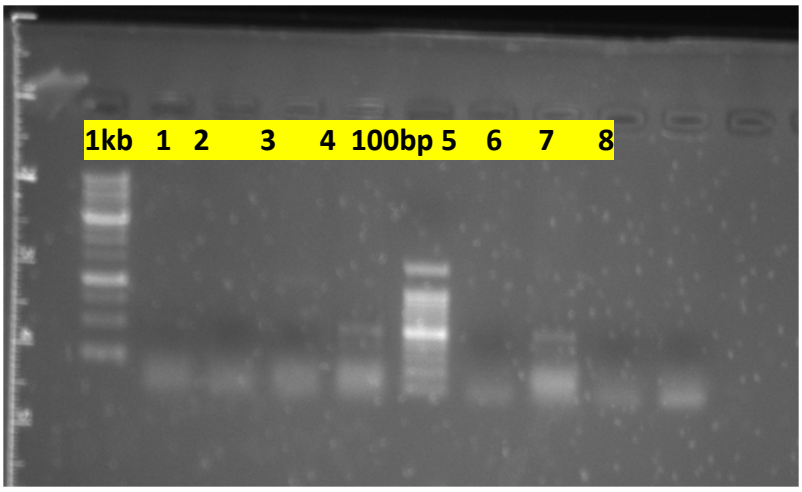
Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony
UPW	6

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	45sec
Final extension	72°C	10min
Hold	4°C	-

Total bp added: 341bp

The excepted product- (p_tet=63, luxI=582, ter=129 total=774bp) 341+774=1115bp
If the Gibson assembly failed (no gene attached to the plasmid) the product will be 192+341=533 bp long.



Results: no positive bands.

Colony PCR 2

08/05/14

New 8 colonies were picked + control

The F primer is 23bp long and 117 bp before the p_tet
The R primer is 23bp long and 178 bp after the terminator

Primers info:

Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

Reaction tube (total volume= 20µl):

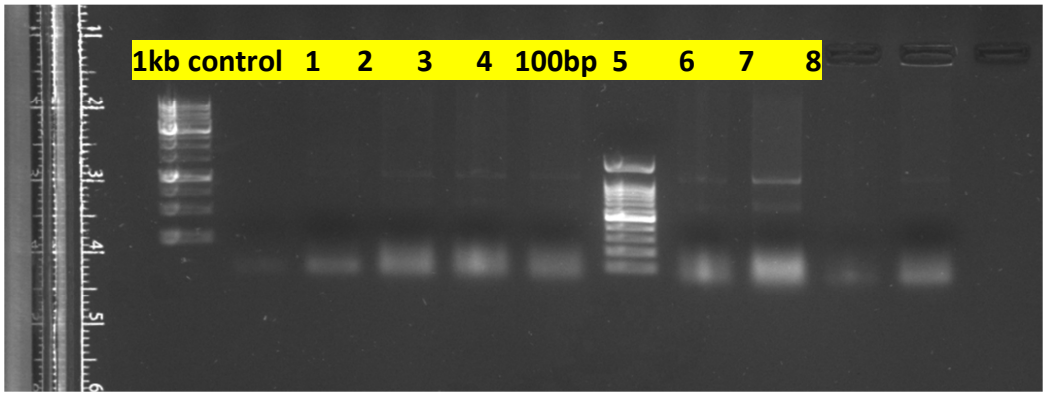
Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony
UPW	6

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	45sec
Final extension	72°C	10min
Hold	4°C	-

Total bp added: 341bp

The excepted product- (p_tet=63, luxI=582, ter=129 total=774bp) 341+774=1115bp
If the Gibson assembly failed (no gene attached to the plasmid) the product will be 192+341=533 bp long.



It seems that colony 2-7 are positive luxI but there is a contamination.

Colony PCR 3

12/05/14

The primers were diluted again since there was probably a contamination.
1 colony was picked from Gibson's plate, colony 2-7 were picked from "colony PCR 2" + control

The F primer is 23bp long and 117 bp before the p_tet
The R primer is 23bp long and 178 bp after the terminator

Primers info:

Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

Reaction tube (total volume= 20µl):

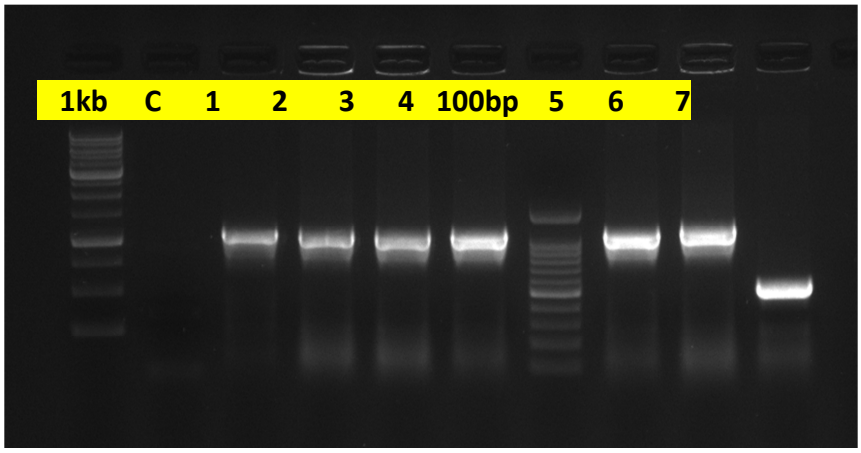
Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony
UPW	6

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	45sec
Final extension	72°C	10min
Hold	4°C	-

Total bp added: 341bp

The excepted product- (p_tet=63, luxI=582, ter=129 total=774bp) 341+774=1115bp
If the Gibson assembly failed (no gene attached to the plasmid) the product will be 192+341=533 bp long.



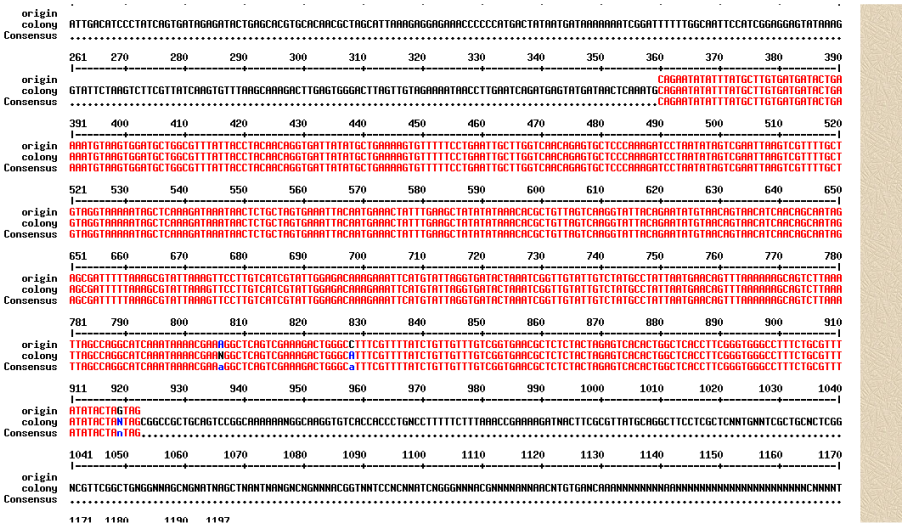
C=control
Colony 1-6 are **positive** luxI
Colony 7 is negative luxI

sequencing

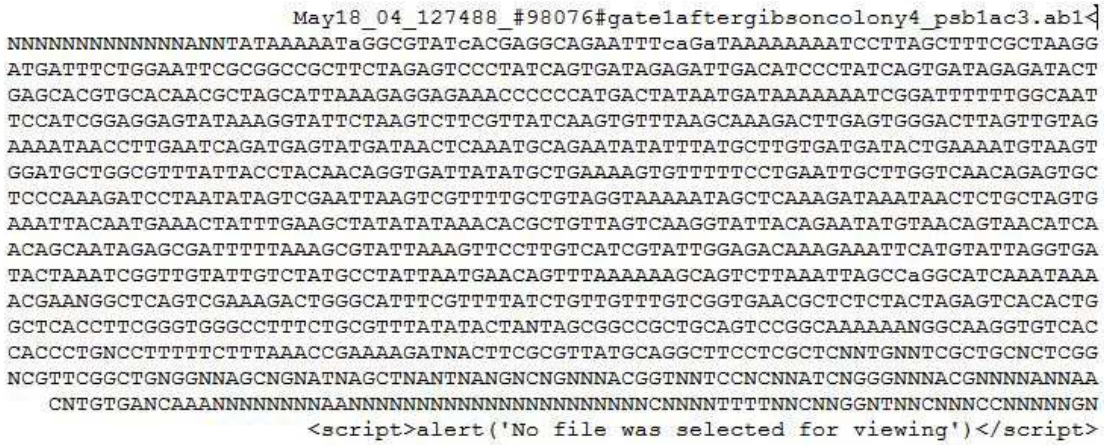
Colony 4 sent to sequencing, glycerol stock at H2 (178.5 ng/µl)

14/05/14

The insertion of luxI succeed:



(the mismatches are in the Terminator which is after the efficiency of the enzyme decreases already sequenced and it was as excepted).



Ptet+luxI+Ter transfer to PSB1C3

29/07/14

Since Prefix and Sufix exist at both plasmid we used restriction enzyme to transfer the insert from PSB1AK3 to psb1c3:

There are two part for the transfer:

1. Cutting the PSB1AK3 with the insert (P_tet+luxI+double Terminator)
   Run it in a gel
   Cut the product (832bp)
   clean it (8.1 ng/µl)

The restriction enzyme reaction:

PSB1AK3+ Insert (from colony 4- see at "sequencing") is 178.5 ng/µl

We need 500 ng of DNA of this reaction:

$$\frac{the\ whole\ plasmid}{insert} = \frac{X}{0.5\ ng} \gg\gg$$
$$\frac{4011\ bp}{832\ bp} = \frac{X}{0.5\ \mu g} \gg\gg X = 2.4\mu g \approx 3\mu g$$

For 3 µg:

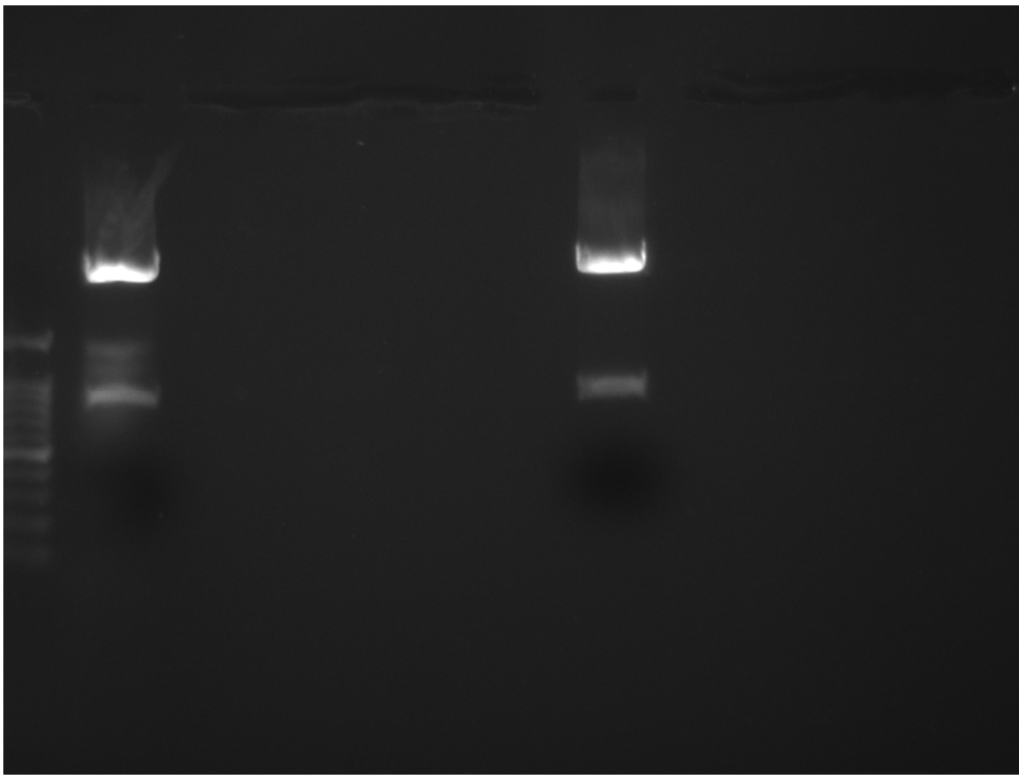
$$V(from\ \text{PSB1AK3} +\ \text{Insert}) = \frac{3\ \mu g}{0.1785\ {}^{\mu g}/_{\mu l}} = 17\mu l$$

The reaction:

μl	
17	DNA (PSB1AK3+INSERT)
0.5	PstI HF
0.5	EcoRI HF
2	Cutsmart
20	total

30 min at 37°C

The gel:
It was contaminated so we cleaned the product from gel
Expected band 801 bp.



For cleaning the volume of the elution: 25 μl
1.5μl for nanodrop: 8.1 ng/μl

2. We cut the PSB1C3 plasmid after the mini prep the concentration was 38 ng/μl:
The reaction:

μl	
15	DNA (PSB1C3)
0.5	PstI HF
0.5	EcoRI HF
2	Cutsmart
2	H_2O
20	total

30 min at 37°C

3. **Clean** from the reaction with the PCR clean protocol-concentration of 13.8 ng/μl.
4. **Ligation** of the pSB1C3 with gate 1 as the insert:

The reaction:

μl	
4	DNA (PSB1C3)
8	DNA (gate 1)
1	T4 enzyme
2	T4 buffer
5	MBW
20	total

2 hr at 30°C

5. Alex did the **transformation** by Heat shock protocol.
6. **Colony PCR:**

6 colonies were picked

The F primer is 23bp long and 117 bp before the p_tet
The R primer is 23bp long and 155 bp after the terminator

Primers info:

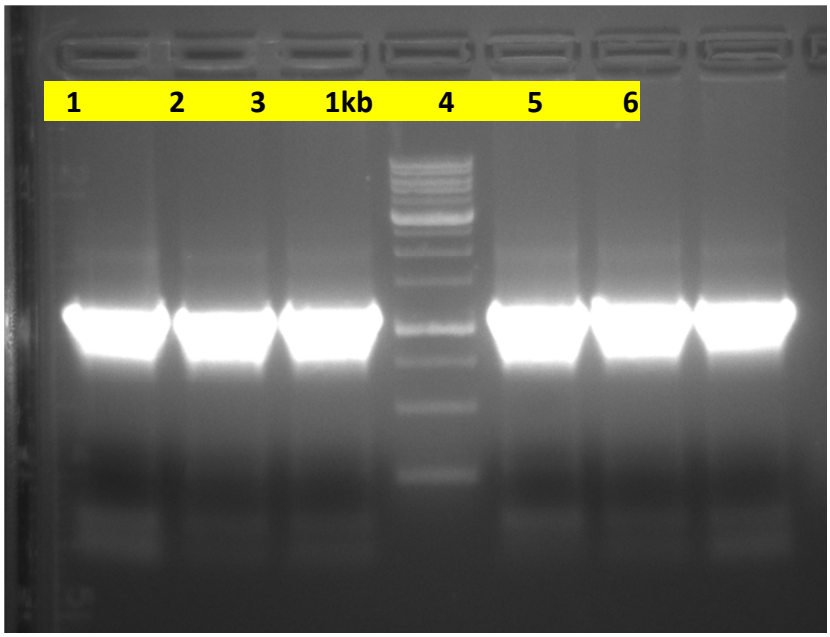
Primer Name	Sequence 5' to 3'	Tm	Comments
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	57	pSB1C3 sequencing of insert S primer
pSB1C3_Ver_AS	CCGTATTACCGCCTTTGAGTGAG	56.7	pSB1C3 sequencing of insert AS primer

Total bp added: 318bp

The excepted product- (p_tet=63, luxI=582, ter=129 total=774bp+ rbs) 318+774+27=1119bp
If the Gibson assembly failed (no gene attached to the plasmid) the product will be 318 bp long.

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	3 min
35 cycles	94°C	30 sec
	55°C	30 sec
	72°C	1:15 min
Final extension	72°C	10min
Hold	4°C	-



Result: all the colonies are positive

Glycerol stock: E8

Pompc+ Gate 1 (pSB1AK3)- amplification

First, the part P_ompc was taken from the distribution kit (psb1c3), transformed to Top10 mini prep (187 ng/µl).

Primers info:

Primer name	Sequence	forward/reversed	Length (bp)	Tm	hair pin	self dimer
p_ompc_gate1_R	aaaaaGCTAGCagtccattctccccaaaaatg	reversed	32	69.3	-0.67	-10.44
pSB1C3_Ver_S	TGCCACCTGACGTCTAAGAAACC	forward		57		

Reaction mix:

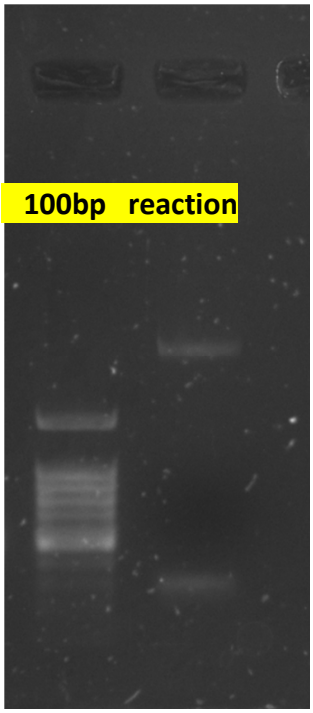
Competent	Volume (µl)
Phusion reaction buffer (X10)	10
dNTP's (10 mM)	1
pSB1C3_Ver_S	2.5
p_ompc_gate1_R	2.5
pSB1C3:ompC (18.7 ng/µl)	1

Phusion hot start II	0.5
DMSO	1.5
UPW	31
Total	50

PCR program

Stage	Temperature (°C)	Time
Initial denaturation	98	30 sec
35 cycles	98 70 72	10 sec 30 sec 15 sec
Final extension	72	10 min
Hold	4	

The product was cleaned and the concentration: 49 ng/µl



The predicted band is 250 bp.
There are two bands - one of them is the product and the second is above 2000 bp.

Pompc+ Gate 1 (pSB1AK3)- promoters replacement

8/9/14

Enzymes digestion

In order to replace P_tet in P_ompc enzyme digestion is required: EcorI + NheI of the P_opmc part and the plasmid of gate 1.

For the Part P_ompC

Reaction tube composition

Component	Volume [µl]
Clean DNA (40 ng/µl)	30
NheI enzyme (HF)	0.5
EcoRI enzyme (HF)	0.5
CutSmart buffer [X10]	4
MBW	5
Total	40

For the plasmid

Reaction tube composition

Component	Volume [µl]
Clean DNA from gate 1 (226 ng/µl)	7
NheI enzyme (HF)	0.5
EcoRI enzyme (HF)	0.5
CutSmart buffer [X10]	2
MBW	10
Total	20

Incubation

The reaction tubes were in 37°C for 30 minutes.

Clean-up

After the restriction we did a PCR clean-up to both tubes and the concentrations we got:

P_ompc- 9.2 ng/µl.
Gate 1 (pSB1AK3) after restriction- 23.4 ng/µl.

Ligation

In order to ligate the G-blocks to the plasmid we did a ligation reaction as follows:

Reaction tube composition

Component	Volume [µl]
Insert	20
Vector	21.5
10X T4 DNA Ligase Buffer	5
T4 DNA Ligase	1
MBW	2.5
Total	50

Incubation

The reaction tubes were in 16°C O/N.

Transformation

5 µL from the ligation reaction were transformed to TOP10 bacteria.
Result: positive.

Colony PCR

To make sure the ligation succeeded, colony PCR was preformed - 3 colonies.
In addition, 1 µl (11 ng/µl) of Gate 1 on pSB1C3 was added to the PCR tube (the same mix).

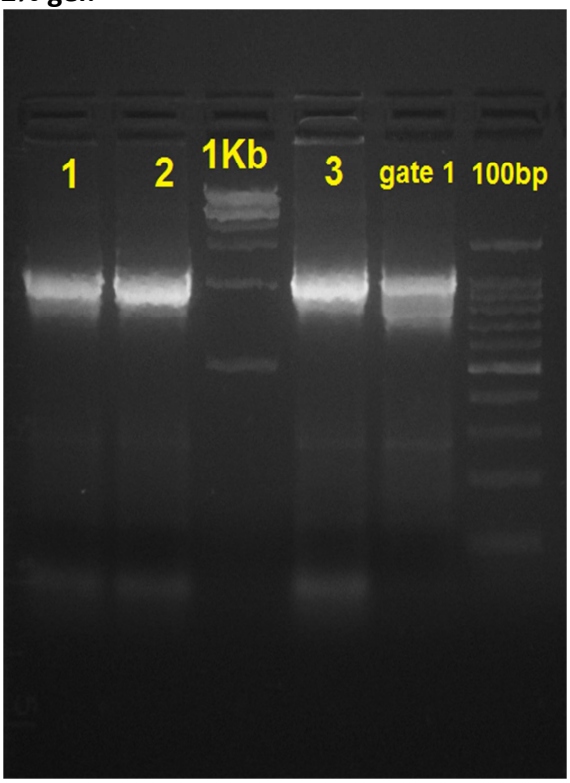
Reaction tube composition

Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony or 1µl of gate 1 on C3
UPW	6

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	45sec
Final extension	72°C	10min
Hold	4°C	-

2% gel:



expected bands:

With p_ompc: 1187 bp (1192)
Original gate 1: 1142 bp

It is all positive, we will send 1-3 to sequencing.

13/9/14

Starter from all 3 colonies.

14/9/14

Mini-prep

15/9/14

Sent to **sequencing** + **Enzymes digestion**
(to transfer to pSB1C3)

Gate 1 with p_ompC transfer to Psb1C3

Enzymes digestion

For the 3 colonies: Cut with EcoRI+PstI the plasmid and gate 1 with p_ompC:

The plasmid was taken from the last reaction.

Gate 1+ p_ompC cut reaction:

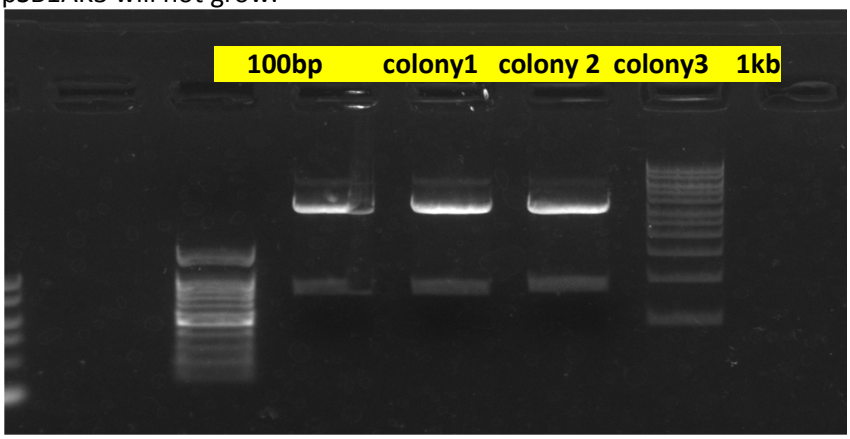
The reaction:

μl	
25	DNA (PSB1AK3+INSERT)
0.5	PstI HF
0.5	EcoRI HF
3	Cutsmart
1	mbw
30	total

incubation

30 min at 37°C

The gel shows positive result from the cut reaction.
We proceed with PCR clean and ligation. Not gel clean cause the growth will be done with CM so colonies with pSB1AK3 will not grow.



After **clean** the concentrations are:
Colony 1: 12.2 ng/ul
Colony 2: 21.5ng/ul
Colony 3: 25.4 ng/ul

According to ligation calculator we need 94 ng of the insert while using 50 ng of the plasmid.

Ligation:

The reaction:

	Colony 1	Colony 2	Colony 3
INSERT	8	4	4
plasmid (PSB1C3)(13.8ng/ul)	4	4	4
mbw	6	10	10
DNA Ligase buffer	2	2	2
T4 DNA Ligase	1	1	1
total	20	20	20

O/N at 16°C

Transformation to Top 10.

Sequencing result: only colony 3 contain p_opmc (colony 1+2 contain p_tet)

```
                    Sep22_02_132490_#105628#pompc-gate1-colony-1_pSB1C3-Ver-S.ab1<
NNNNNNNNGNNTNNNNNTATAAAAATAGGCGTATcacgaggcAgAATTTcaGatAAAAAAAATCCTTAGCTTTCGCTAAG
GATGATTTCTGGAATTCGCGGCCGCTTCTAGAGTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATAC
TGAGCACGTGCACAACGCTAGCATTAAAGAGGAGAAACCCCCCATGACTATAATGATAAAAAAATCGGATTTTTTGGCAA
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GAAAATAACCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATGCTTGTGATGATACTGAAAATGTAAG
TGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTTTCCTGAATTGCTTGGTCAACAGAGTG
CTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGTAGGTAAAAATAGCTCAAAGATAAATAACTCTGCTAGT
GAAATTACAATGAAACTATTTGAAGCTATATATAAACACGCTGTTAGTCAAGGTATTACAGAATATGTAACAGTAACATC
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                                        GNNNNNNNNNNNNNNNNNCNNNNNCNNNNNAANCNCAAGGNAANNNN
                                <script>alert('No file was selected for viewing')</script>
```

17/9/14

Colony PCR

*only for colony 3 from the sequencing result above: 4 colonies were picked.

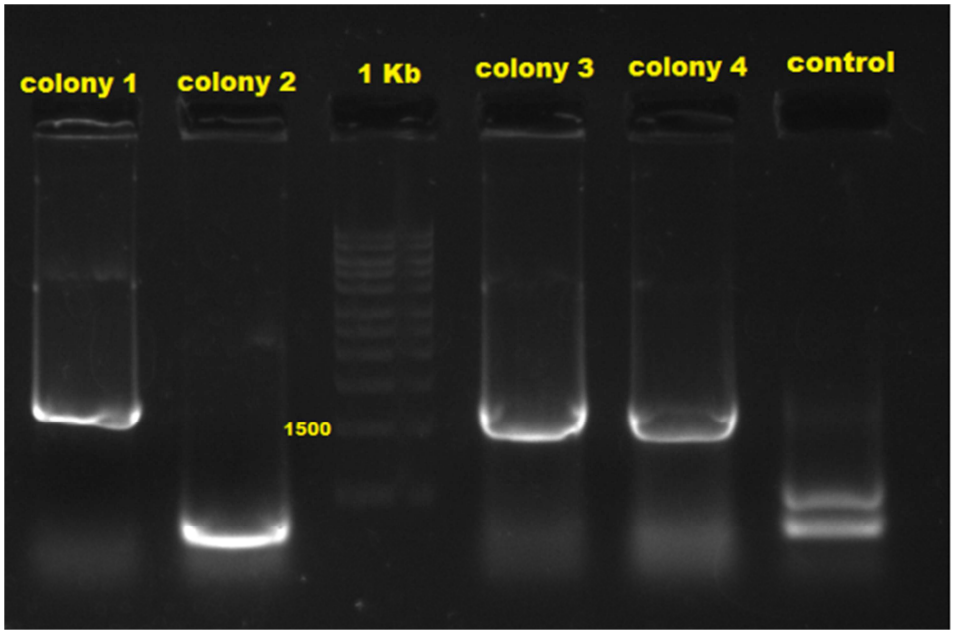
Reaction tube:

Component of sample	Volume (µl)
Taq ready mix (2X)	10
Forward primer (10mM)	2
Reversed primer (10mM)	2
Template DNA	1 colony
UPW	6

PCR program:

Stage	Temp.	time
Initial denaturation	94°C	
35 cycles	94°C	30sec
	56°C	30sec
	72°C	60sec
Final extension	72°C	10min
Hold	4°C	-

Expected band: 1187 bp.



Results:

Colony 2 - the ligation didn't succeed

Colony 1,3,4- succeed.

Starter from colonies 1,3,4

After mini prep of colony 3 the concentration is 395 ng/ul.

Colony 3 is in -80°C in the box: Igem 2014 B, at position G4.

Gate 2

Getting parts from the distribution kit and transformation

29.2.14

Distribution kit

For assembly of gate 2 we took the following parts from the iGEM distribution kit:

Part name	Length	Part number
Lac promoter	200bp	BBa_R0010
LuxR	781bp	BBa_C0062
Double terminator	129bp	BBa_B0015

Transformation

2 µL from every part were transformed to TOP10 bacteria. The bacteria were plated on the appropriate antibiotics.

Transformation results and startes

30.2.14

Transformation results

The transformation of the parts to Top10 bacteria worked- we saw colonies on both plates- 100µl and rest.

Starters

We made starters for the bacteria containing the parts.

Mini preps

30.2.14

Mini preps for Plac, LuxR and double terminator

We did a mini prep for each starter from yesterday.

Concentrations

Sample name	Concentration
pSB1A2-Plac	91.9 ng/µl
pSB1AK3-ter	144.5 ng/µl
pSB1A2-LuxR	114 ng/µl

Amplification of Plac, double terminator and pSB1C3

1.3.14

Amplification of Plac promoter

In order to assemble gate 2 we needed to amplify the promoter from the distribution kit.

Reaction tube composition

Component	Volume [µl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: Plac F [10µM]	2.5
Reverse primer: Plac R [10µM]	2.5
Template: pSB1A2-Plac [91.9 ng/µl]	5.5
Phusion hot start	0.5
DMSO	1.5
UPW	26.5
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	5
Annealing	64	30 sec	5
Extension	72	1 min	5
Denaturation	98	10 sec	30
Annealing	70	30 sec	30
Extension	72	1 min	30
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈240 bp.

Amplification of the double terminator

In order to assemble gate 2 we needed to amplify the double terminator from the distribution kit.

Reaction tube composition

Component	Volume [µl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: ter F [10µM]	2.5
Reverse primer: ter R [10µM]	2.5
Template: pSB1AK3-ter [144.5 ng/µl]	3.5
Phusion hot start	0.5
DMSO	1.5
UPW	28.5
Total	50

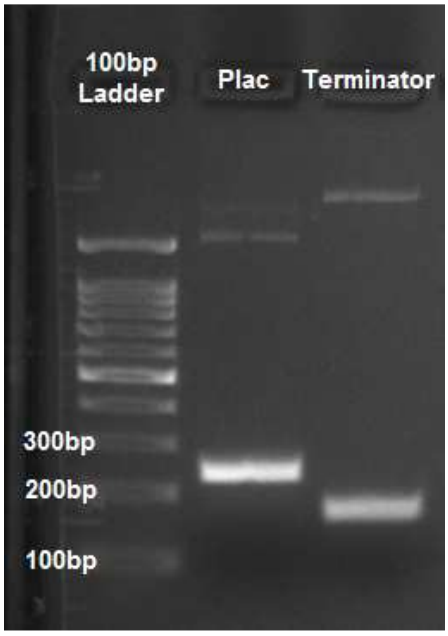
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	5
Annealing	64	30 sec	5
Extension	72	1 min	5
Denaturation	98	10 sec	30
Annealing	70	30 sec	30
Extension	72	1 min	30
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈170 bp.

After the PCR reactions we ran the product in 1% agarose gel.

The gel image



From the gel image we saw that the PCR reactions for Plac and terminator worked.

Clean-up

We cleaned the PCR products using PCR clean-up kit.

Sample name	Concentration
Plac after clean-up	51 ng/μl
Double terminator after clean-up	69 ng/μl

Amplification of pSB1C3- first try

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 F [10μM]	2.5
Reverse primer: 1C3 R [10μM]	2.5
Template: pSB1C3 [97 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	71	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈2040 bp.

In the gel images we saw too much unspecific products.

Amplification of pSB1C3

2.3.14

Amplification of pSB1C3- second try

In the last attempt to amplify the plasmid we got too many unspecific products, so this time we decided to change the reaction annealing temperature.

Reaction tube composition

Component	Volume [µl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 F [10µM]	2.5
Reverse primer: 1C3 R [10µM]	2.5
Template: pSB1C3 [97 ng/µl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	69	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈2040 bp.

Again, in the gel images we saw too much unspecific products.

Amplification of pSB1C3- third try

This time we wanted to try a gradient PCR with two annealing Temperature: 55°C and 60°C. But again we saw in the gel unspecific products.

One of the problems in our reaction was that the template concentration was too high.

Amplification of pSB1C3

3.3.14

Amplification of pSB1C3- fourth try

This time we made dilution of our plasmid sample to concentration of 2 ng/µl.
Gradient PCR was done with two annealing Temperature: 55°C and 60°C.

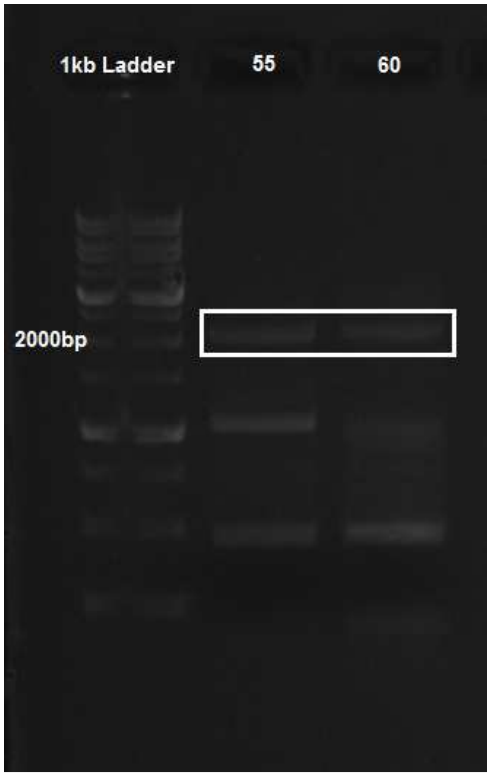
Reaction tube composition

Component	Volume [µl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 F [10µM]	2.5
Reverse primer: 1C3 R [10µM]	2.5
Template: pSB1C3 [2 ng/µl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	55/60	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The gel image



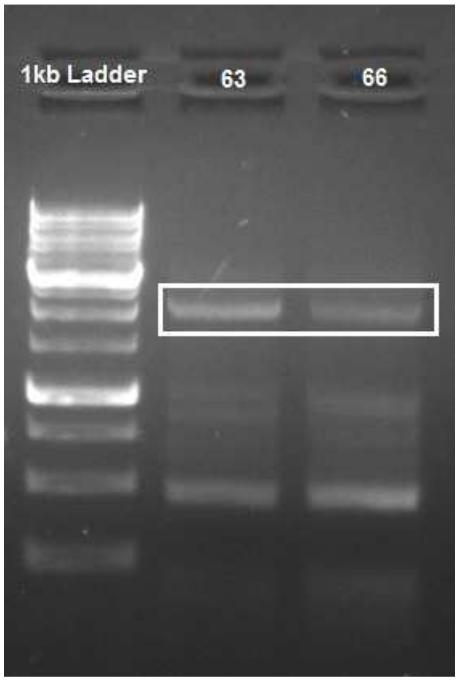
From the gel we again that the reaction wasn't specific.

We tried to do a gel extraction, but the plasmid concentration we received after the clean-up was around 0.

Amplification of pSB1C3- fifth try

We decided to try a gradient PCR with different annealing temperature: 63°C and 66°C.

The gel image



After we saw the gel image we decided to do a gel extraction. The concentration we got was 10.7 ng/µl.

Gibson assembly

4.3.14

Gibson assembly

We did a Gibson assembly in order to build the part pSB1C3-Plac-ter.

Transformation

5.3.14

Transformation

We preformed a transformation of the Gibson assembly product (pSB1C3- Plac-ter) to TOP10 bacteria.

The bacteria were plated on CM plates: 100 µl and rest.

Transformation results

6.3.14

Transformation results

After an overnight incubation colonies have grown on both plates.

Colony PCR

11.3.14

Colony PCR

We did a colony PCR to verify the insertion of Plac-ter into the plasmid pSB1C3. Overall we checked 5 colonies in the colony PCR.

Reaction tube composition

Component	Volume [µl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10µM]	2
Reverse primer: pSB1C3 ver AS [10µM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	45 sec	35
Final extension	72	10 min	-
Hold	4	-	-

- The primers for the PCR are the pSB1C3 sequencing primers.

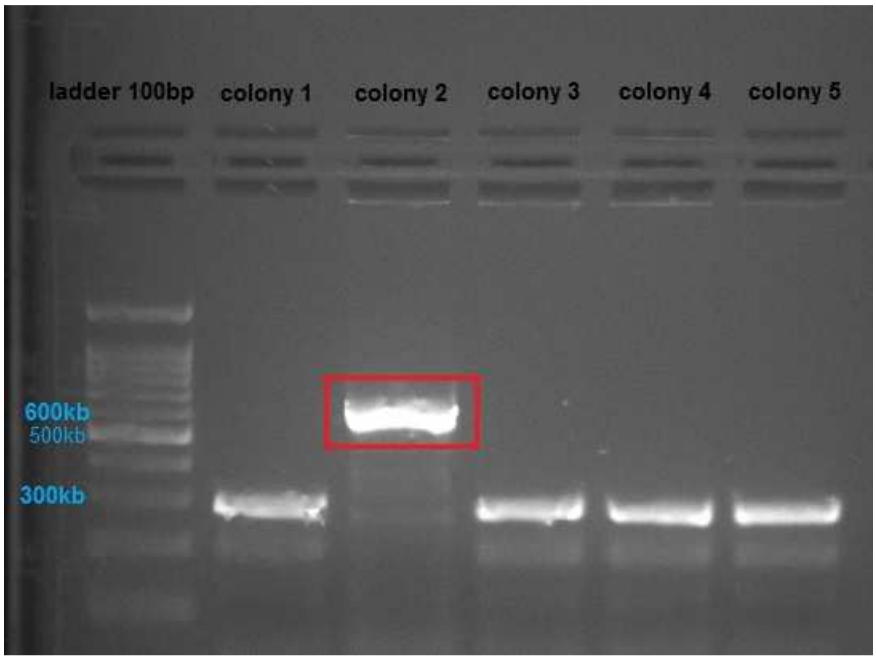
- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

- pSB1C3 Ver AS:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

The Insert length is 350 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+350+23+23=668 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp

The gel image

We made 2% agarose gel in order to check the PCR products.



From the gel image we saw that only colony 2 was positive.

Starter

12.3.14

Starter

We Made a Starter from colony 2, using the back-up plate.

Glycerol stock and mini prep

13.3.14

Glycerol stock and mini prep

Before starting the mini prep we made a glycerol stock. The stock is stored in the clear box, 1G.

After the mini prep the DNA concentration was 142.5 ng/µl.

Sequencing

16.3.14

Sequencing

We sent the sample pSB1C3-Plac-ter to sequencing.

Sequencing results and starters

19.3.14

Sequencing results

We got sequencing results not as expected. The problem was probably the sequencing primer for the plasmid pSB1C3.

Starters

Two starters were made from the pSB1C3-Plac-ter glycerol stock in order to send the sample to sequencing again with new diluted primer.

Mini preps

20.3.14

Mini preps

We did a mini-prep for each starter. The concentrations we got- 84.5 and 180 ng/µl.

Sequencing

23.3.14

Sequencing

We sent the sample pSB1C3-Plac-ter to sequencing with new diluted primers (forward and reverse).

Enzymes digestion

Enzymes digestion

We have done a restriction enzymes (EcoRI and PstI) reaction to verify the insertion of Plac-ter into the plasmid pSB1C3.

Reaction tube composition

Component	Volume [µl]
pSB1C3-Plac-ter after m.p [180 ng/µl]	8.3
PstI HF enzyme	0.5
EcoRI HF enzyme	0.5
Buffer CutSmart	2
MBW	8.7
Total	20

Negative control reaction tube composition

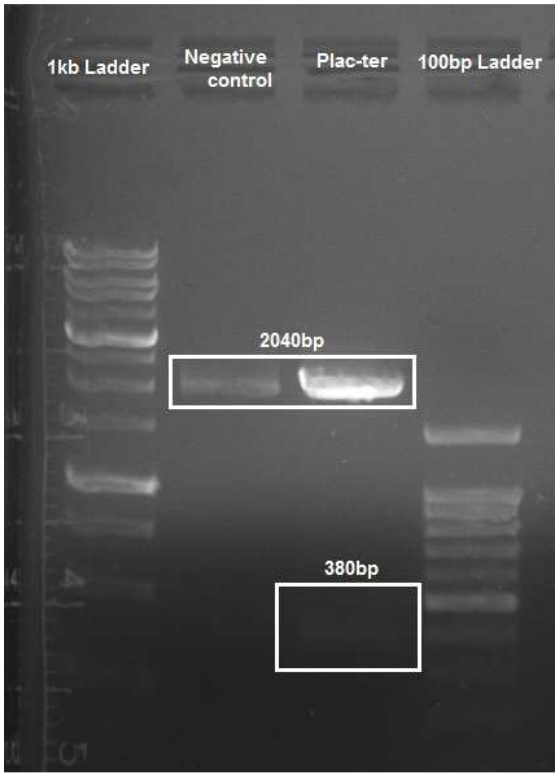
Component	Volume [µl]
pSB1C3 after m.p [9.7 ng/µl]	3.6
PstI HF enzyme	0.5
EcoRI HF enzyme	0.5
Buffer CutSmart	2
MBW	13.4
Total	20

For the Plac-ter sample we were supposed to get a 380bp segment and a 2040bp segment.
For the negative control we were supposed to get only the 2040bp segment.

Incubation

The reaction tube was in 37°C for 30 minutes.

Gel image



In the gel image we saw that for the Plac-ter sample we got two bands, meaning the Gibson assembly worked.

Sequencing results

26.3.14

Sequencing results

The sequencing of pSB1C3-Plac-ter was successful.

Amplification of LuxR and pSB1C3-Plac-ter

16.4.14

Amplification of LuxR

In order to assemble gate 2 we needed to amplify LuxR from the distribution kit.

Reaction tube composition

Component	Volume [µl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: LuxR F [10µM]	2.5
Reverse primer: LuxR R [10µM]	2.5
Template: pSB1A2-LuxR [2 ng/µl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

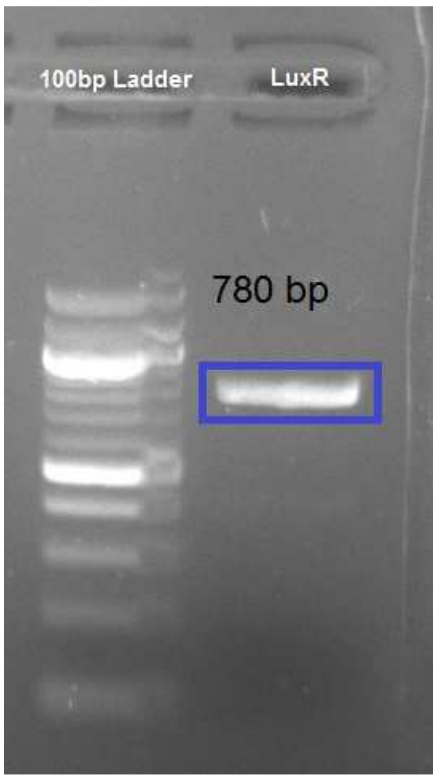
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	65	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈780 bp.

After the PCR reaction we ran the product in 2% agarose gel.

The gel image



From the gel image we saw that the PCR reaction for LuxR amplification worked.

Clean-up

We cleaned the PCR product using PCR clean-up kit.

Sample name	Concentration
LuxR after clean-up	31 ng/µl

Amplification of pSB1C3-Plac-ter

Reaction tube composition

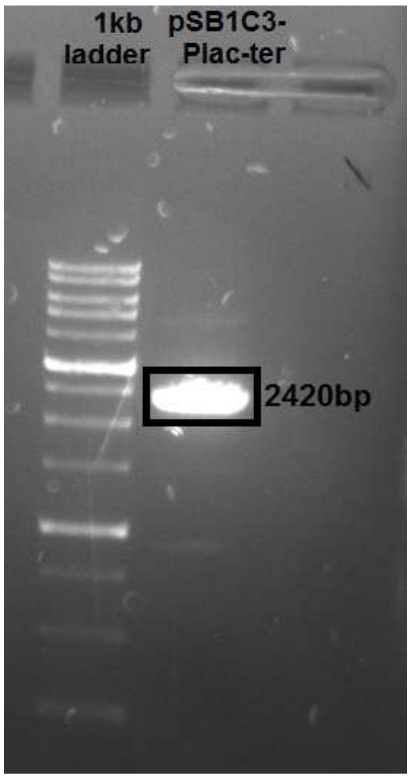
Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: Plac-ter F [10μM]	2.5
Reverse primer: Plac-ter R [10μM]	2.5
Template: pSB1C3-Plac-ter [2 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	69	30 sec	35
Extension	72	1:30 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: ≈2420 bp.

The gel image



From the gel image we saw that the PCR reaction for pSB1C3-Plac-ter amplification worked.

Clean-up

We cleaned the PCR product using PCR clean-up kit.

Sample name	Concentration
pSB1C3-Plac-ter after clean-up	52 ng/µl

Gibson assembly

17.4.14

Gibson assembly

We did a Gibson assembly in order to build the part pSB1C3-Plac-LuxR-ter.

Transformation

20.4.14

Transformation

We preformed a transformation of the Gibson assembly product (pSB1C3- Plac-LuxR-ter) to TOP10 bacteria.
The bacteria were plated on CM plates: 100 µl and rest.

Transformation results

21.4.14

Transformation results

After an overnight incubation colonies have grown on both plates.

Colony PCR

22.4.14

Colony PCR

We did a colony PCR to verify the insertion of Plac-LuxR-ter into the plasmid pSB1C3. Overall we checked 6 colonies in the colony PCR.

Reaction tube composition

Component	Volume [µl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10µM]	2
Reverse primer: pSB1C3 ver AS [10µM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:30 min	35
Final extension	72	10 min	-
Hold	4	-	-

- The primers for the PCR are the pSB1C3 sequencing primers.

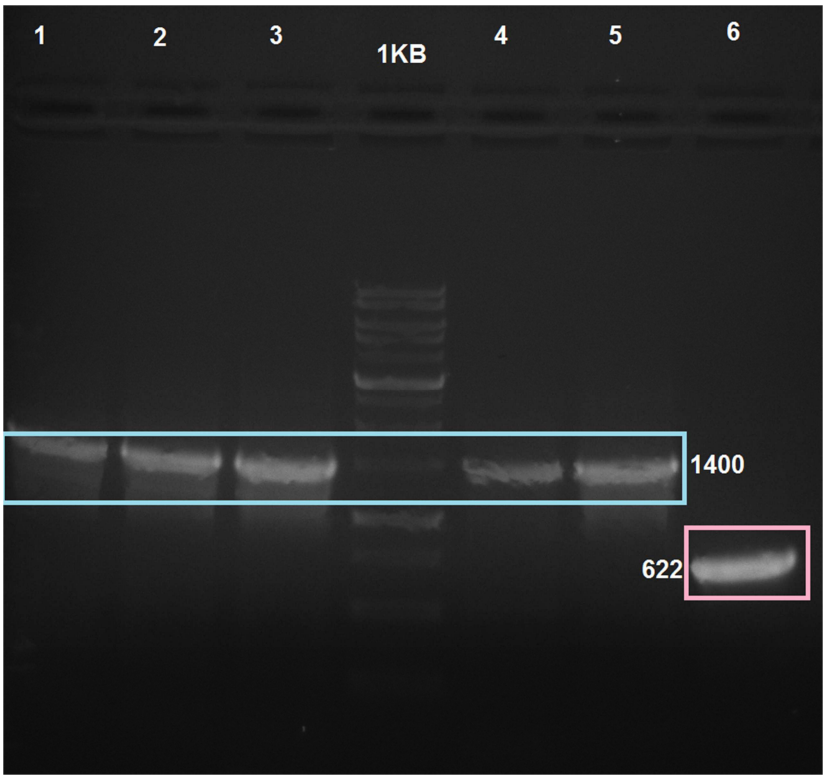
- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

- pSB1C3 Ver AS:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

The Insert length is 350 bp.
Positive result: total DNA length expected in gel, plasmid with insert ≈1400 bp.
Negative result: Total DNA length expected in gel, plasmid without insert ≈620 bp.

The gel image

We made 1% agarose gel in order to check the PCR products.



From the gel image we saw that colonies 1-5 were positive.

Starter

23.4.14

Starter

We Made a Starter from colony 4, using the back-up plate.

Glycerol stock and mini prep

24.4.14

Glycerol stock and mini prep

Before starting the mini prep we made a glycerol stock. The stock is stored in the clear box, 3G.

After the mini prep the DNA concentration was 67 ng/µl.

Sequencing

27.4.14

Sequencing

We sent the sample pSB1C3-Plac-LuxR-ter to sequencing.

Sequencing results

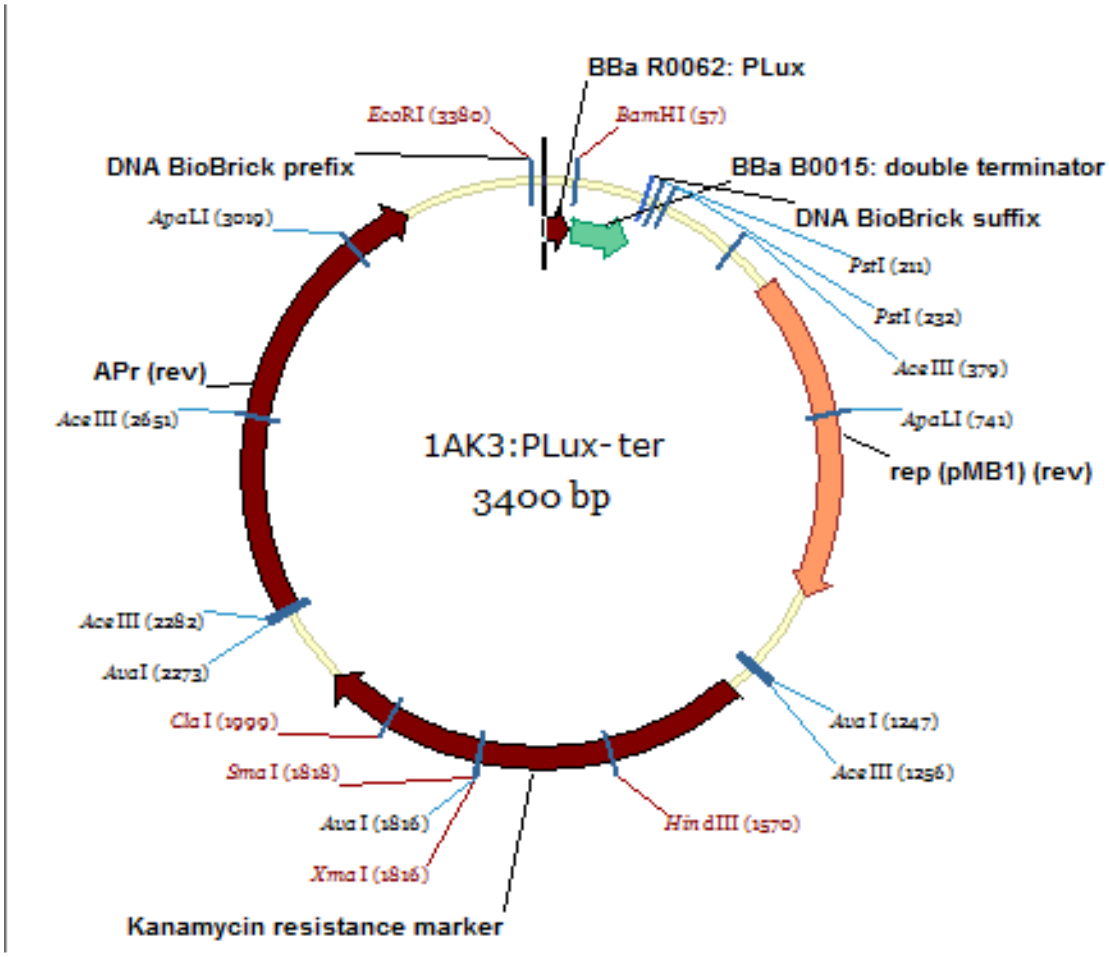
30.4.14

Sequencing results

The sequencing of pSB1C3-Plac-LuxR-ter was successful.

Gate 3

pSB1AK3:pLux-ter assembly



BBa_R0062: PLux | BamHI | BBa_B0015: double terminator

```
ACCTGTAGGA TCGTACAGGT TTACGCAAGA AAATGGTTTG TTATAGTCGA ATAAAGGATC CCCAGGCATC AAATAAAACG AAAGGCTCAG TCGAAAGACT
TGGACATCCT AGCATGTCCA AATGCGTTCT TTTACCAAAC AATATCAGCT TATTTCCTAG GGGTCCGTAG TTTATTTTGC TTTCCGAGTC AGCTTTCTGA
```

BBa_B0015: double terminator | DNA BioBrick suffix

```
GGGCCTTTCG TTTTATCTGT TGTTTGTCGG TGAACGCTCT CTACTAGAGT CACACTGGCT CACCTTCGGG TGGGCCTTTC TGCGTTTATA TACTAGTAGC
CCCGGAAAGC AAAATAGACA ACAAACAGCC ACTTGCGAGA GATGATCTCA GTGTGACCGA GTGGAAGCCC ACCCGGAAAG ACGCAAATAT ATGATCATCG
```

Primers order:

primer	Forward /reversed	length	% GC	first cycles Tm	Tm	hair pin	self dimer	hetero dimer	name
GCA AGA AAA TGG TTT GTT ATA GTC GAA TAA AGG ATC CCC AGG CAT CAA ATA AAA CG	forward	56	37.5	61	72	-0.6	-10.76	-9.29	1AK: plux f s.1
GTA AAC CTG TAC GAT CCT ACA GGT CTC TAG AAG CGG CCG CGA ATT C	reversed	46	54.8	61	72	-5.67	-22.78		1AK: plux r s.1

Reverse PCR reaction

07/03/2014

Primers:

Primer name	**forward/ reversed**	**First cycle Tm**	**Tm**	**Add DMSO?**	**Reaction number**
1AK:plux f s.1	forward	61	72	yes	1
1AK:plux r s.1	reversed	61	72	yes	

Reaction mix:

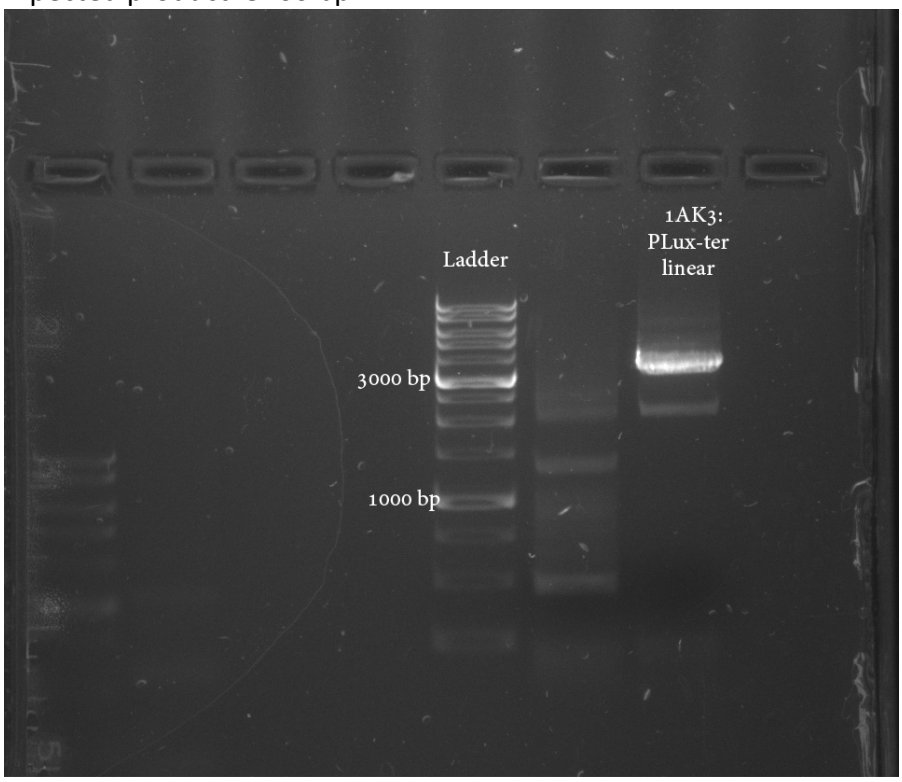
component	Volume[µl]	In?
phusion reaction buffer(x5)	10	V
dNTPs(10 mM)	1	V
1AK:plux f s.1forward primer	2.5	V
1AK:plux r s.1reverse primer	2.5	V
pSB 1AK3:ter (144.5 ng/µl)	5	V
Phusion hot start II	0.5	V
DMSO	1.5	V
UPW	27	V
tot	50	+

PCR program

stage	Temp C^0	time
Initial denaturation	98	30 sec
35 cycles	98	10 sec
	69	30 sec
	72	1 min
Final extension	72	10 min
hold		

Run gel agarose 1% for product validation

Expected product: 3400 bp



Clean products (clean PCR Kit)

Part name	1AK3:Plux-ter (lin)
date	7.3.2014
concentration	19 [ng/µl)
-20 ^{0}C storage	iGEM Gate 3+M13 \D6

Extraction from gel

Blunt ligation

Reaction mix:

08/03/2014

Reaction content	Volume [µl]	In?
T4 ligase buffer(x10)	2.5	v
T4 kinase (PNK)	1	v
1AK3:Plux-ter (lin) 19[ng/µl]	6	v
PEG 4000 (50%)	2.5	v
DDW	13	v
total	25	+

Incubate at 37 0C for 1 hr starting at 14:20
Inactivation 65 0C for 20 min
Ligation started at 15:50 for 2 hr at 25 0C in dry both.

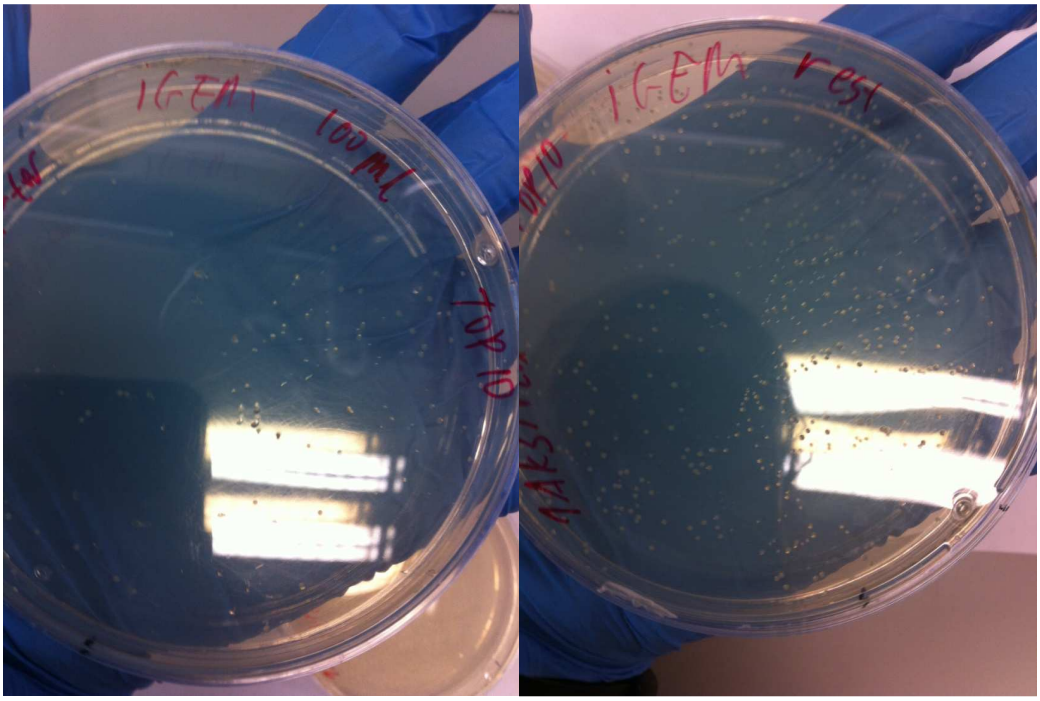
Storage in -20 ^{0}C

Part name	1AK3:Plux-ter (cir)
date	8.3.2014
-20 ^{0}C storage	iGEM Gate 3+M13\D7

Transformation to top 10

20 min frost on ice 100 mL
5 mL of 1AK3:Plux-ter (cir)
30 min on ice
1 min heat shock 42 ^{0}C
2 min on ice

incubation overnight.



Colony PCR

Primers:

10/03/2014

Primer name	**forward/ reversed**	**First Tm**	**Tm**	**Add DMSO?**
pLux f	**forward**	50	63	yes
ter r	**reversed**	50	63	yes

Reaction mix: make total mix and then divide 20 [µl] to each of 7 PCR tube

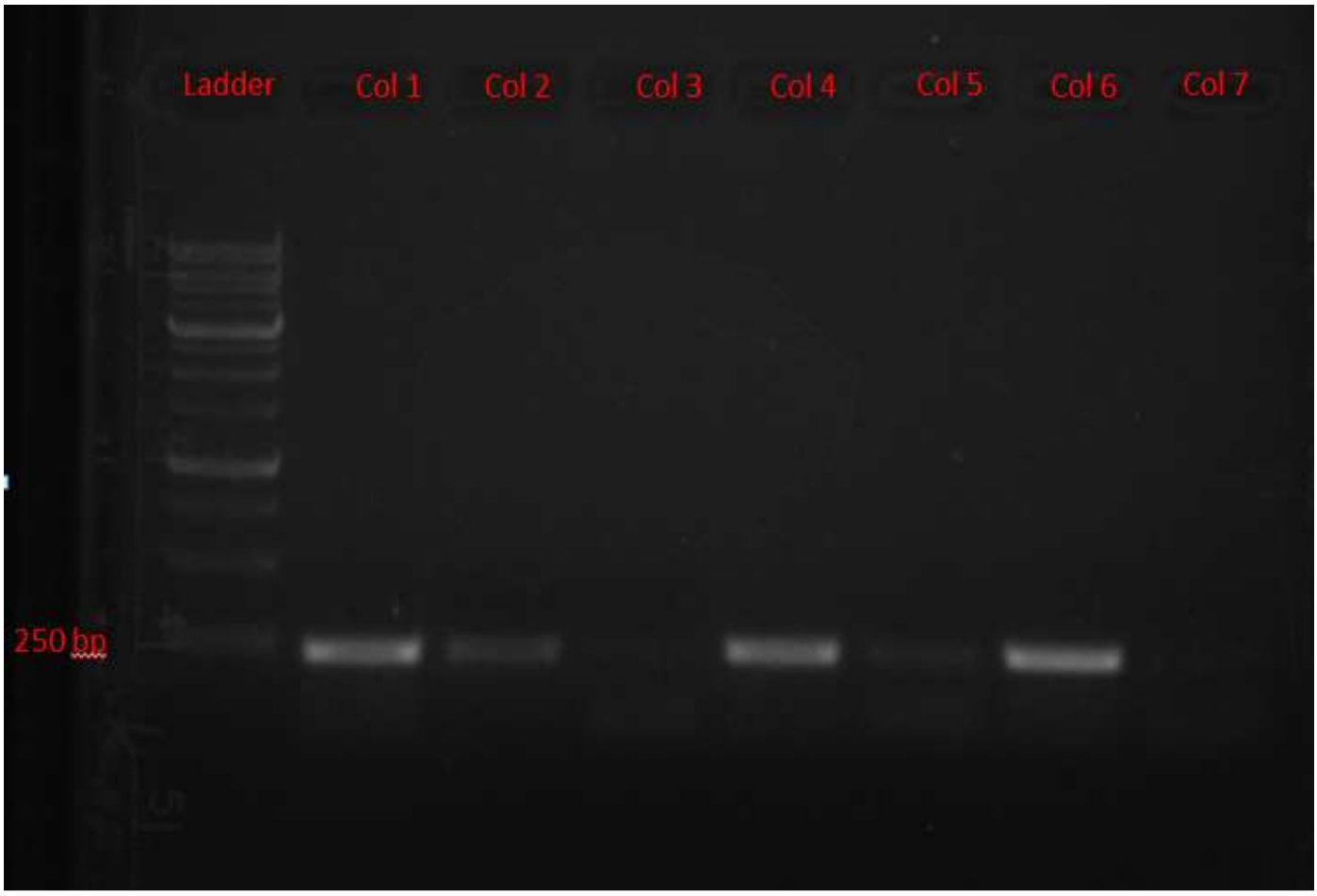
component	Volume[µl] for 1 colony	For total mix	In?
Taq ready mix (x2)	10	70	
DMSO	0.5	3.5	
pLux f (10 ng/µl)	1	7	
ter r (10 ng/µl)	1	7	
1AK3:PLux-ter	colony	----	
UPW	7.5	52.5	
tot	20	140	

PCR program

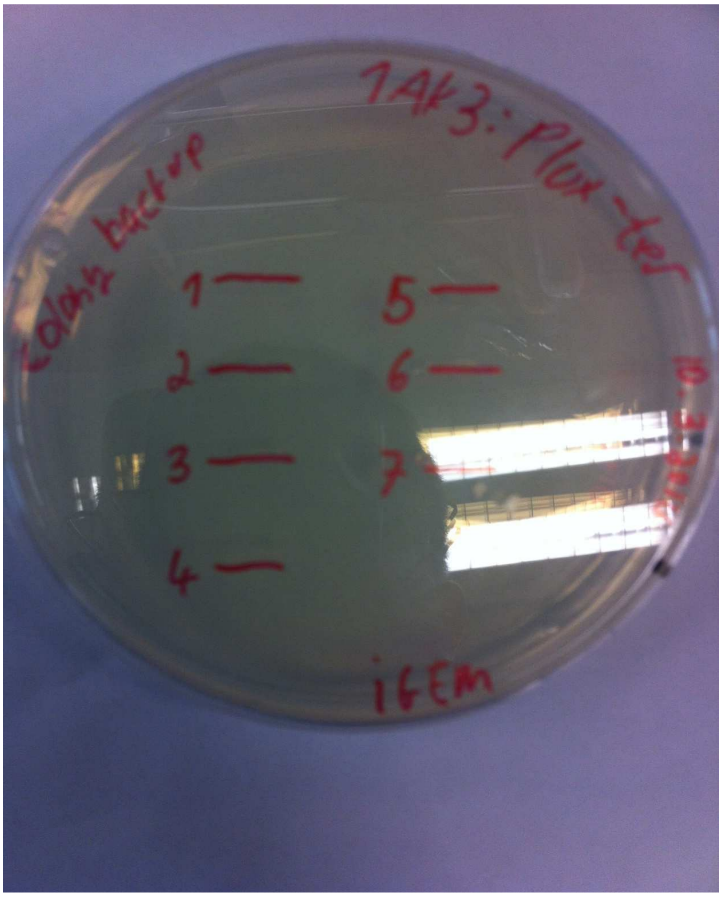
stage	Temp C^0	time
Initial denaturation	94	3 min

35 cycles	94	30 sec
	56	30 sec
	72	1 min
Final extension	72	10 min
hold	4	

Run gel agarose 1% for product validation
Expected product : 250 bp



Positive colonies: 1,4,6



Miniprep

13/03/2014

From positive colony make starter overnight

Make glycerol stock

Part name	1AK3:Plux-ter col.1	1AK3:Plux-ter col.6
date	13.3.2014	13.3.2014
Cell type	Top 10	Top 10
resistance	CM	CM
-80 ^{0}C storage box name	iGEM clear	iGEM clear
Location	F7	F8

Minprep

Part name	1AK3:Plux-ter col.1	1AK3:Plux-ter col.6
date	13.3.2014	13.3.2014

concentration	298[ng/μl)	371[ng/μl)
-20 ^{0}C storage	iGEM Gate 3+M13\D8	iGEM Gate 3+M13 \D9

Sequencing

17.03.2014

Send to sequencing

24.03.2014

Sequencing results

Results from CLUSTALW analysis (11:22 24/03/2014)

```
col1            ATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGAGACCTGTAG
col6            ATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGAGACCTGTAG
ref             ----------------------------------------------------ACCTGTAG
                                                                     ********

col1            GATCGTACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAAGGATCCCCAGGCA
col6            GATCGTACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAAGGATCCCCAGGCA
ref             GATCGTACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAAGGATCCCCAGGCA
                ************************************************************

col1            TCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTC
col6            TCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTC
ref             TCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTC
                ************************************************************

col1            GGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTA
col6            GGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTA
ref             GGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTA
                ************************************************************

col1            TATACTAGTAGCGGCCGCTGCAGT--------------------CCGGCAAAAAA--GG
col6            TATACTAGTAGCGGCCGCTGCAGT--------------------CCGGCAAAAAA--GG
ref             TATACTAGTAGCGGCCGCTGCAGTACTAGTAGCGGCCGCTGCAGTCCGGCAAAAAAACGG
                ************************                   ***********  **
```

Stage 1 have succeeded

from 1AK3 to 1C3

Transfer Plux-ter from 1AK3 to 1C3 by Gibson assembly

PCR reactions

03/04/2014

Plux-ter extraction from 1AK3

Primer name	forward/ reversed	Tm	Add DMSO?	Reaction number
1C3 f st.2	forward	69	yes	2
1C3 r st.2	reversed	69	yes	

Reaction mix:
Dilute
pSB 1C3 (97 ng/µl) x50 to about 2 ng/µL final concentration

component	Volume[µl]	In?
phusion reaction buffer(x5)	10	
dNTPs(10 mM)	1	
1C3 f st.2 forward primer	2.5	
1C3 r st.2 reverse primer	2.5	
pSB 1C3 (2 ng/µl)	5.5	
Phusion hot start II	0.5	
DMSO	1.5	
UPW	26.5	
tot	50	

PCR program

stage	Temp C^0	time
Initial denaturation	98	30 sec
35 cycles	98	10 sec
	69	30 sec
	72	2 min
Final extension	72	10 min
hold		

Run gel agarose 1% for product validation
Expected product:
Plux-ter: 250bp
pSB1C3: 2070 bp

Amplification of pSB1C3

Primer name	**forward/ reversed**	**First cycle Tm**	**Tm**	**Add DMSO?**	**Reaction number**
pLux f st.2	forward	61	61	yes	1
ter r st.2	reversed	59	59	yes	

Reaction mix:
Dilute 1AK3:Plux-ter col1 (298 ng/μl) x100 to about 2 ng/μL final concentration

component	Volume[μl]	In?
phusion reaction buffer(x5)	10	
dNTPs(10 mM)	1	
pLux f st.2 forward primer	2.5	
ter r st.2 reverse primer	2.5	
pSB 1AK3:Plux-ter col1 (2 ng/μl)	5	
Phusion hot start II	0.5	
DMSO	1.5	
UPW	27	
tot	50	

PCR program

stage	Temp C^0	time
Initial denaturation	98	30 sec
5 cycles	98	10 sec
	55	30 sec
	72	1 min
30	98	10 sec
	69	30 sec
	72	30 sec
Final extension	72	10 min
hold		

Clean products(clean PCR Kit)

Part name	Plux-ter (lin)
date	27/03/2014
concentration	39 [ng/µl)
-20 ^{0}C storage	iGEM Gate 3+M13 \E7
by	PCR cleanup kit

Part name	pSB1C3 (lin)
date	04/04/2014
concentration	15 [ng/µl)
-20 ^{0}C storage	iGEM Gate 3+M13 \E8
by	Gel extraction

Comments: twice the gel extraction have failed , thus after the extraction from gel make sure that the band completely transferred to the micro-centrifuge tube by observe the gel again under UV.

Gibson assembly

04/04/2014

Insertion of plux-ter to pSB1C3 by Gibson assembly

Calculation of the concentrations:

$$Plux-ter\left[\frac{pmol}{\mu l}\right]=\frac{Plux-ter\left[\frac{ng}{\mu l}\right]\times 10^3}{size\times 650}=\frac{39\left[\frac{ng}{\mu l}\right]\times 10^3}{250\times 650}=0.24\left[\frac{pmol}{\mu l}\right]$$

$$1C3\left[\frac{pmol}{\mu l}\right]=\frac{1C3\left[\frac{ng}{\mu l}\right]\times 10^3}{size\times 650}=\frac{15\left[\frac{ng}{\mu l}\right]\times 10^3}{2070\times 650}=0.01\left[\frac{pmol}{\mu l}\right]$$

Reaction mix:

component	Volume[µl]	In?
Gibson assembly master mix(x2)	15	+
pSB1C3 (0.05pmol)	5	+
Plux-ter (lin) (0.15 pmol)	0.5	+
UPW	9.5	+
tot	30	

Incubation at 50 ^{0}C for 1 hr.

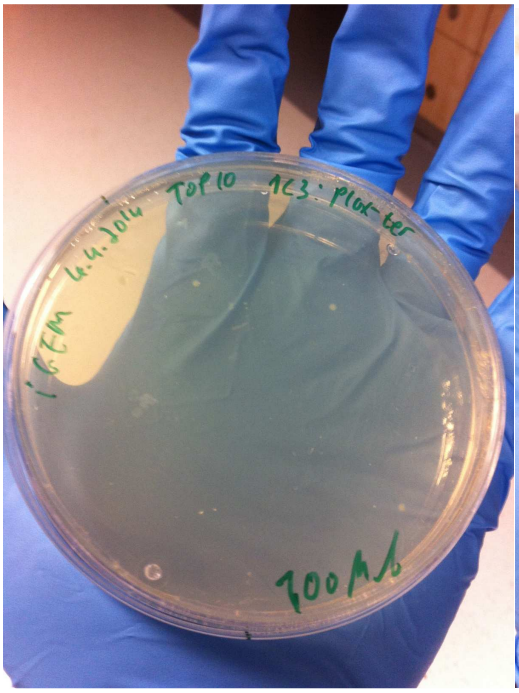
Store in -20 ^{0}C

Part name	1C3:Plux-ter (cir)
date	04/04/2014
-20 ^{0}C storage	iGEM Gate 3+M13 \E7

Transformation to top 10

20 min frost on ice 100 mL
5 mL of 1C3:Plux-ter (cir)
30 min on ice
1 min heat shock 42 ^{0}C
2 min on ice

incubation overnight.



Colony PCR

Attempt 1

Primer name	**forward/ reversed**	**Tm**	**Add DMSO?**
pLux f st.2	**forward**	55	yes
ter r st.2	**reversed**	55	yes

Reaction mix: make total mix and then divide 20 [µl] to each of 7 PCR tube

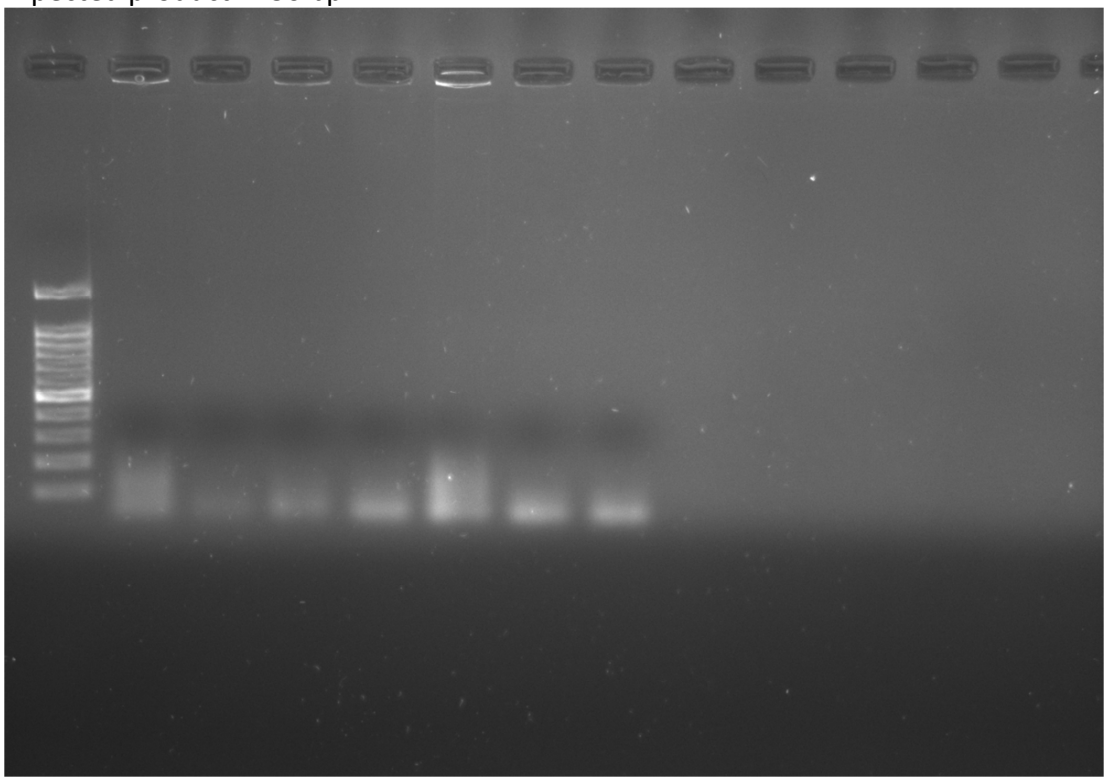
component	Volume[µl] for 1 colony	For total mix	In?
Taq ready mix (x2)	10	70	+
DMSO	0.5	3.5	+
pLux f st.2 (10 ng/µl)	1	7	+
ter r st.2 (10 ng/µl)	1	7	+
1C3:PLux-ter	colony	----	+
UPW	7.5	52.5	+
tot	20	140	

PCR program

stage	Temp C^0	time
Initial denaturation	94	3 min
35 cycles	94	30 sec
	55	30 sec
	68	1 min
Final extension	68	10 min
hold	4	

Run gel agarose 1% for product validation

Expected product : 250 bp



Positive colonies: NO

08/04/2014

Attempt 2

Primer name	**forward/ reversed**	**Tm**	**Add DMSO?**
pLux f st.2	**forward**	55	yes
ter r st.2	**reversed**	55	yes

Reaction mix: make total mix and then divide 20 [µl] to each of 7 PCR tube

component	Volume[µl] for 1 colony	For total mix	In?
Taq ready mix (x2)	10	100	
DMSO	0.5	5	
pLux f st.2 (10 ng/µl)	1	10	
ter r st.2 (10 ng/µl)	1	10	
1C3:PLux-ter	colony	----	
UPW	7.5	75	
tot	20	200	

PCR program

stage	Temp C^0	time
Initial denaturation	94	30 sec
5 cycles	94	10 sec
	55	30 sec
	72	30 min
30	98	10 sec
	69	30 sec
	72	30 sec
Final extension	72	10 min
hold		

Run gel agarose 1% for product validation

Expected product : 250 bp

Positive colonies:

05/04/2014

Miniprep

09/04/2014

From positive colony make starter overnight

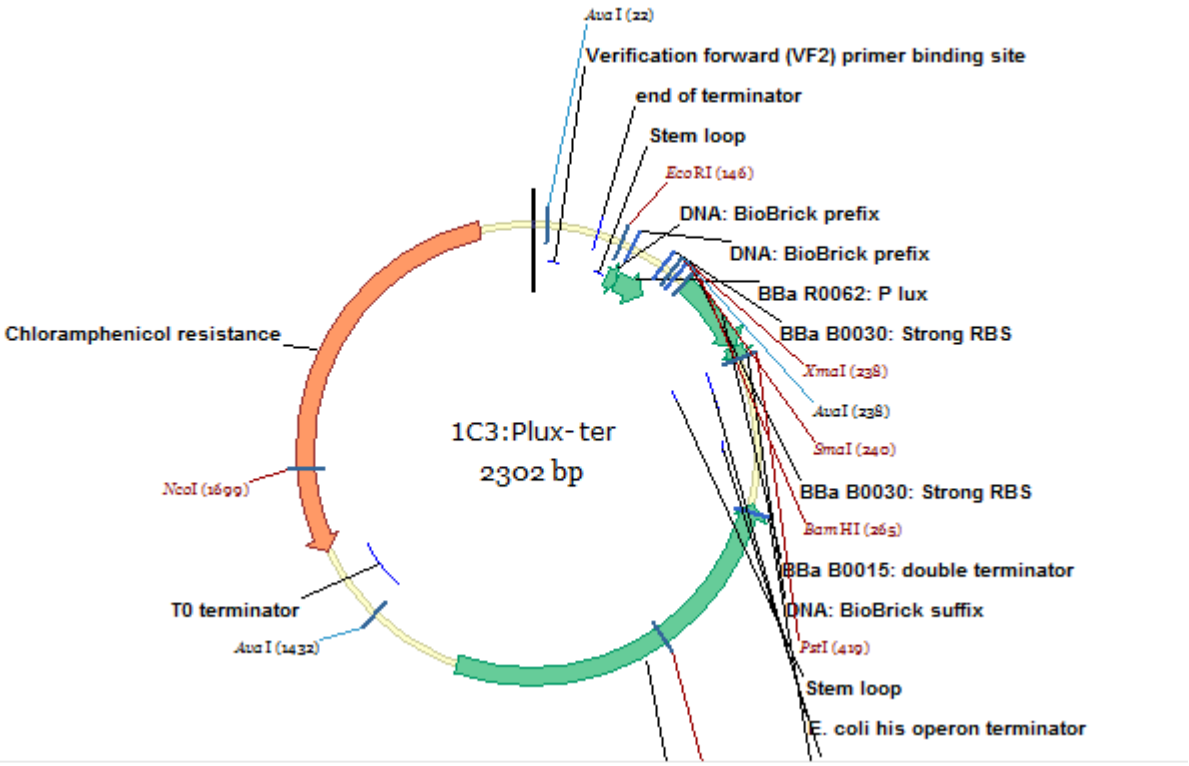
Make glycerol stock

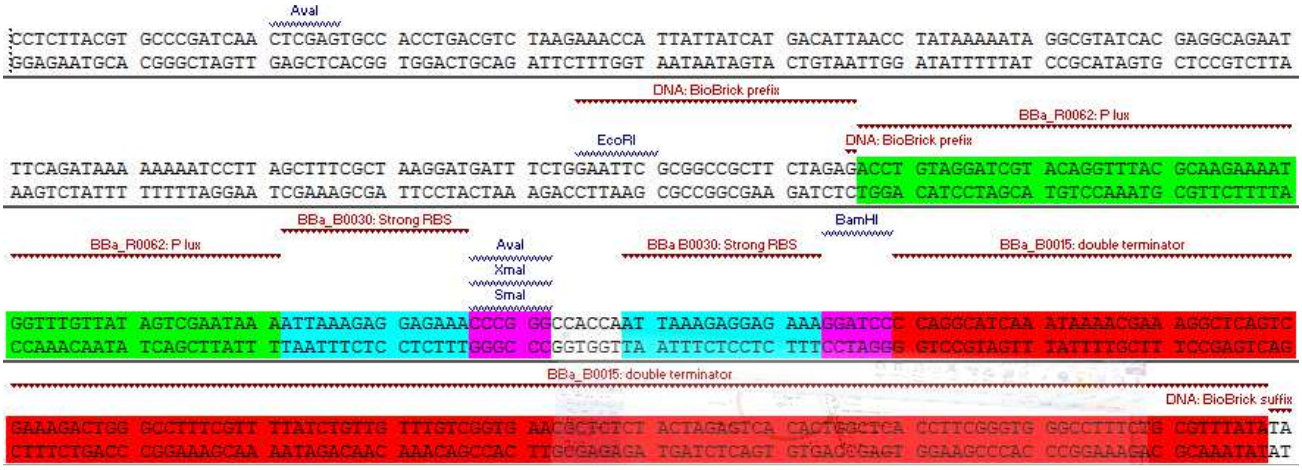
Part name	1C3:Plux-ter st 2
date	09/04/2014
Cell type	Top 10
resistance	CM
-80 ^{0}C storage box name	Igem 2014 clear
Location	F9

Minprep

Part name	1C3:Plux-ter
date	09/04/2014
concentration	35
-20 ^{0}C storage	iGEM Gate 3+M13

Add RBS to pSB1C3:pLux-ter





Glycerol stock st 2 miniprep

20/05/2014

Starter from glycerol stock 1C3:Plux-ter st 2 at 17:35

Minprep

Part name	1C3:Plux-ter st.2
date	21.5.2014
concentration	65 [ng/µl)
-20 ^{0}C storage	iGEM Tal & Rica \ F2

Reverse PCR reaction

21/05/2014

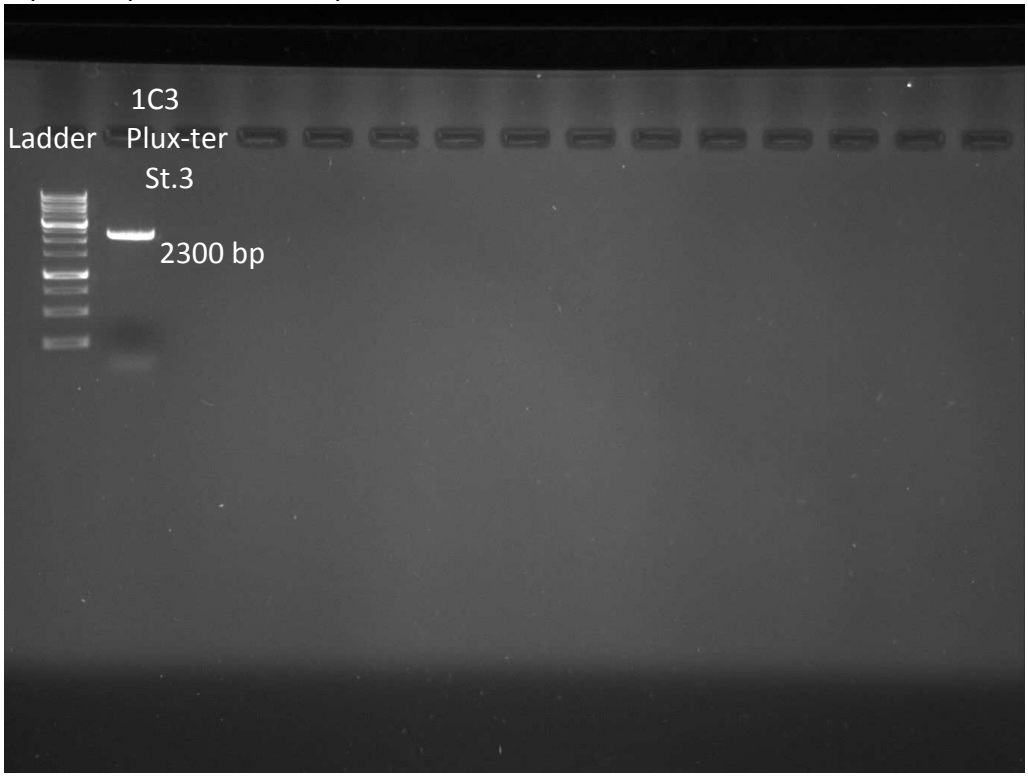
Primer name	forward/ reversed	First cycle Tm	Tm	Add DMSO?
1C3 f st.3	forward	50	62	yes
1C3 r st.3	reversed	50	62	yes

Dialut 1C3:Plux-ter from 65 to 2ng/mL by adding 3[ml] to 87 [ml] MBW
Reaction mix:

PCR program (total time 1:36 hr, start at 11:40)

stage	Temp C^0	time
Initial denaturation	98	30 sec
35 cycles	98	10 sec
	69	30 sec
	72	1:20 min
Final extension	72	10 min
hold	4	-----

Run gel agarose 1% for product validation
Expected product: 2300bp



Clean products (clean PCR Kit)

Part name	1C3:Plux-ter (lin)
date	21/05/2014
concentration	53 (ng/µl)
-20 ^{0}C storage	iGEM Gate 3+M13\ F6

Extraction by PCR cleanup kit

Blunt ligation

23/05/2014

Reaction content	Volume [µl]	In?
T4 ligase buffer(x10)	2.5	v
T4 kinase (PNK)	1	v
1C3:Plux-ter (lin) 53[ng/µl]	2	v
PEG 4000 (50%)	2.5	v
DDW	17	v
total	25	+

Incubate at 37 0C for 1 hr starting at
Inactivation 65 0C for 20 min
Ligation started at 15:50 for O\N at 16 ^{0}C in dry both.

Storage in -20 ^{0}C

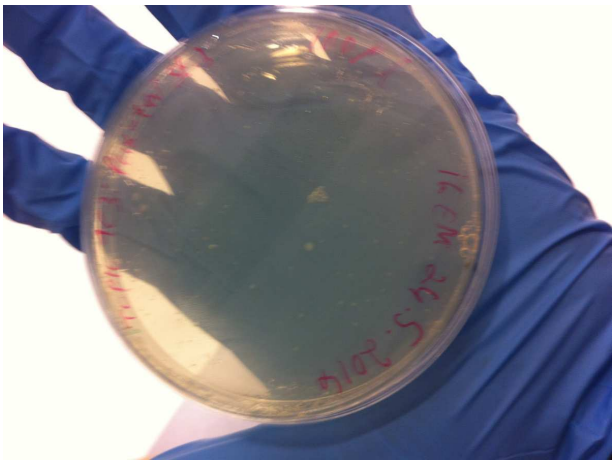
Part name	1C3:Plux-ter (cir)
date	24/05/2014
-20 ^{0}C storage	iGEM Gate 3+M13 \ F7

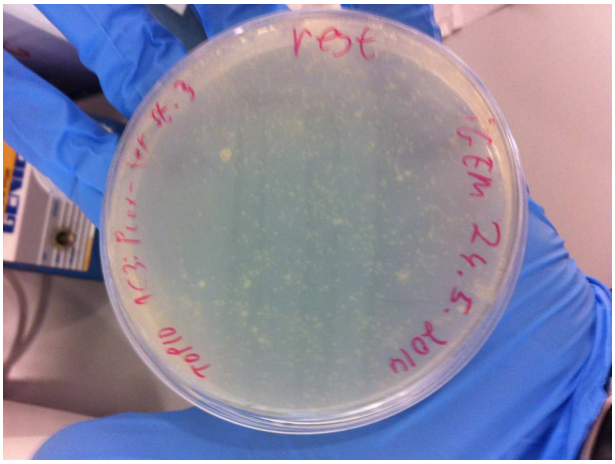
Transformation to top 10

24/05/2014

20 min frost on ice 100 µL
5 µL of 1C3:Plux-ter (cir)
30 min on ice
1 min heat shock 42 ^{0}C
2 min on ice

incubation overnight.





Restriction enzyme double cut scanning

26/05/2014

Make starter from 7 colonies at 26/05/2014 17:05
Incubate overnight.
Miniprep

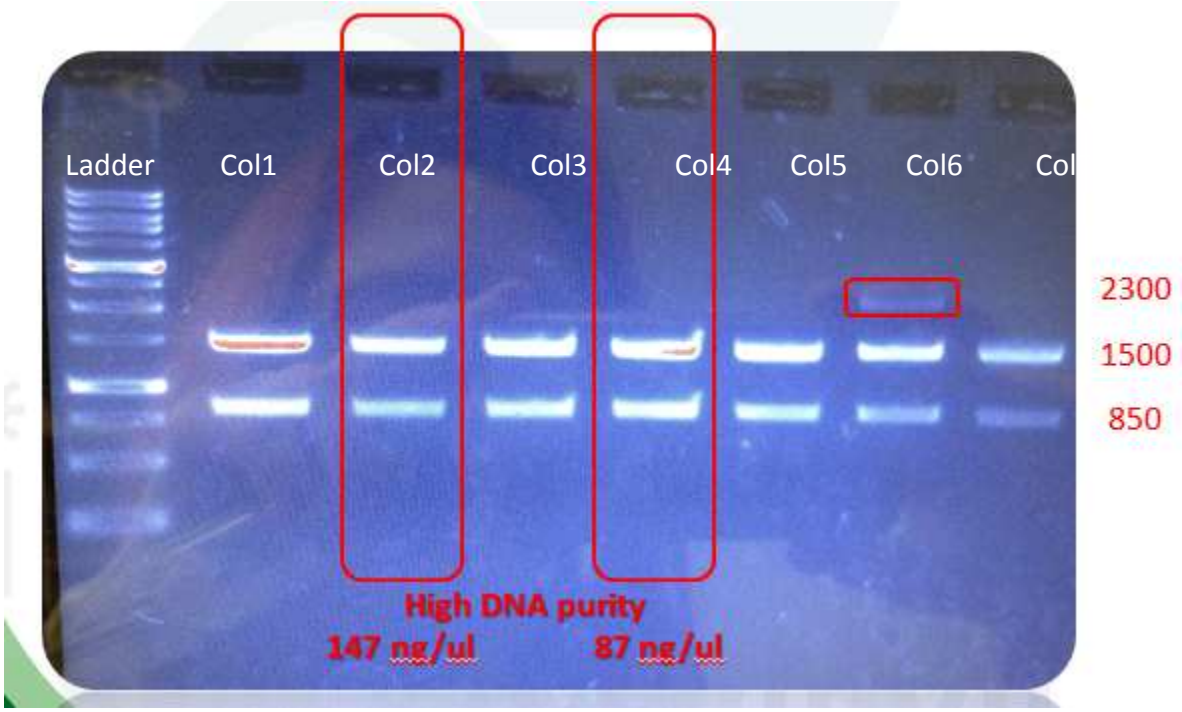
Part name	1C3:Plux-ter st.3	1C3:Plux-ter st.3	1C3:Plux-ter st.3	1C3:Plux-ter st.3	1C3:Plux-ter st.3	1C3:Plux-ter st.3	1C3:Plux-ter st.3
colony	1	2	3	4	5	6	7
date	26/05/2014	26/05/2014	26/05/2014	26/05/2014	26/05/2014	26/05/2014	26/05/2014
concentration	83 (ng/ml)	147 (ng/ml)	114 (ng/ml)	87 (ng/ml)	92 (ng/ml)	119 (ng/ml)	108 (ng/ml)
-20 ^{0}C storage	iGEM Gate 3+M13\ F6						

Extraction by PCR cleanup kit

t1C3:Plux-ter st.3	Consentration [ng/ml]	Volume [ml]			Cutsmart Buffer		total
Colony num.	Final 1000 ng	DNA	Nco I	Xma I	(x10)	MBW	
1	83	13	0.5	0.5	2	4	20
2	147	7	0.5	0.5	2	10	20
3	114	9	0.5	0.5	2	8	20
4	87	12	0.5	0.5	2	5	20
5	92	11	0.5	0.5	2	6	20
6	119	9	0.5	0.5	2	8	20
7	108	10	0.5	0.5	2	7	20

Incubate for 1 hr at 37 ^{o}C dry bath
Cool on ice.

Run gel agarose 1% for product validation
Expected product positive: 850,1500
Expected product negative: 2300



Positive colonies: 1,2,3,4,5,7

It seems that colony 6 is impure and originate from 2 clones. An other possibility is that the clone resaved 2 plasmids . Technical problem might occurred, parasail digestion.

Glycerol stock

05/06/2014

From positive colony make starter overnight

Colony 2 and colony 4

Make glycerol stock

Part name	1C3:Plux-ter st.3 col.2	1C3:Plux-ter st.3 col.4
date	05/06/2014	05/06/2014
Cell type	Top 10	Top 10
resistance	CM	CM
-80 ^{0}C storage box name	iGEM clear	iGEM clear
Location	G4	G5

Sequencing results

12/06/2014

```
ACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAAATTAAAGAGGAGAAACCCG
ACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAA------------------
AANTNGNNNAAACTNNNNAGGGANNGGNNNAGACGAAAAAAC----------------
*                 * **   *    ** **** ***

GGCCACCAATTAAAGAGGAGAAAGGATCCCCAGGCATCAAATAAAACGAAAGGCTCAGTC
----------------------GGATCCCCAGGCATCAAATAAAACGAAAGGCTCAGTC
----------------------ATNTNTCAATAAACCCTTTAGGGAAATANNCCANNTT
                        *  * *   * *   **    * *  *    *

GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCA
GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCA
TTNNNNNTNACACGCCACATCTTGCGAATATATGT--GTAGAAACTGCCGGAAATCGTCG
      *    *     * *  *   * * ***  **  *  **  *    *  ***
```

RBS-SmaI site-RBS deleted
Sequencing determine stage 3 failed

```
AGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGAGACCTGTAGGATCGT
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AGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGAGACCTGTAGGATCGT
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ACAGGTTTACGCAAGAAAATGGTTTGTTATAGTCGAATAAAATTAAAGAGGAGAAACCCG
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************************************************************

GGCCACCAATTAAAGAGGAGAAAGGATCCCCAGGCATCAAATAAAACGAAAGGCTCAGTC
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GGCCACCAATTAAAGAGGAGAAAGGATCCCCAGGCATCAAATAAAACGAAAGGCTCAGTC
************************************************************

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GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCA
************************************************************

CACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAGT
CACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAGT
CACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGTAGCGGCCGCTGCAGT
************************************************************

CCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTA
CCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTA
CCGGCAAAAAAGGGCAAGGTGTCACCACCCTGCCCTTTTTCTTTAAAACCGAAAAGATTA
************************************************************
```

Succeeded!

Insertion of GFP

PCR reactions

Amplification of pSB1C3

Reaction mix:
Dilute
pSB 1C3 (147 ng/μl) x74 to about 2 ng/μL final concentration by add 1.5 μL to 98.5 μL UPW

component	Volume[μl]	In?
phusion reaction buffer(x5)	10	+
dNTPs(10 mM)	1	+
1C3 f st.2 forward primer	2.5	+
1C3 r st.2 reverse primer	2.5	+
pSB 1C3 (2 ng/μl)	5	+
Phusion hot start II	0.5	+
DMSO	1.5	+
UPW	27	+
tot	50	V

PCR program

stage	Temp C^0	time
Initial denaturation	98	30 sec
35 cycles	98	10 sec
	69	30 sec
	72	1:20 min
Final extension	72	10 min
hold		

GFP Mut3 extraction from K133

15/06/2014

Primer name	forward/ reversed	First cycle Tm	Tm	Add DMSO?	Reaction number
GFP f st.4	forward	55	69	yes	1
GFP r st.4	reversed	55	69	yes	

Reaction mix:

Dilute GFP Mut3 (344 ng/μl) to about 2 ng/μL final concentration by add 1 μL to 170 μL UPW

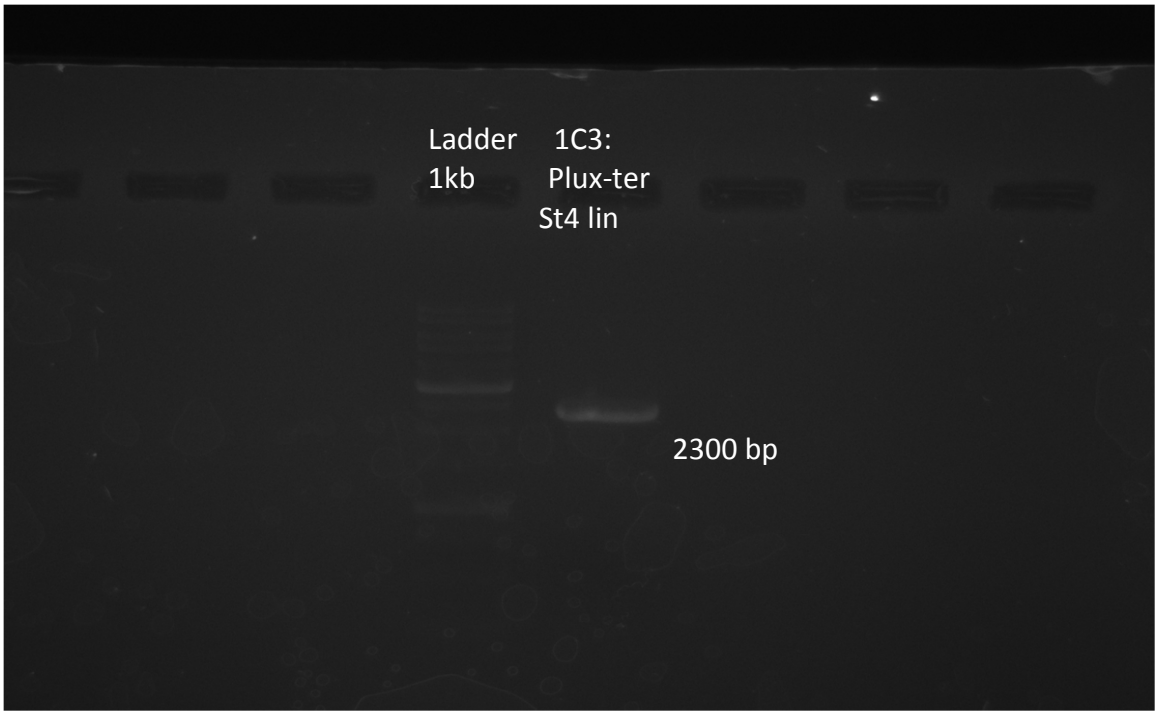
component	Volume[μl]	In?
phusion reaction buffer(x5)	10	+
dNTPs(10 mM)	1	+
GFP f st.4 forward primer	2.5	+
GFP r st.4 reverse primer	2.5	+
GFP mut3 (2 ng/μl)	5	+
Phusion hot start II	0.5	+
DMSO	1.5	+
UPW	27	+
tot	50	V

PCR program

stage	Temp C^0	time
Initial denaturation	98	30 sec
5 cycles	98	10 sec
	55	30 sec
	72	30 sec
30	98	10 sec
	69	30 sec
	72	30 sec
Final extension	72	10 min
Hold		

04/06/2014

Run gel agarose 1% for product validation
Expected product:
GFP: 765 bp
pSB1C3: 2300 bp



Clean products (clean PCR Kit)

Part name	1C3:Plux-ter st.3 col.2 (lin)
date	04/06/2014
concentration	113[ng/µl)
-20 ^{0}C storage	Gate3+M13\H6
by	PCR cleanup kit

Part name	GFP mut3 (lin)
date	16/06/2014
concentration	35 [ng/µl)
-20 ^{0}C storage	Gate3+M13\H7
by	PCR cleanup kit

Gibson assembly

12/06/2014

Insertion of plux-ter to pSB1C3 by Gibson assembly
Calculation of the concentrations:

$$GFP\left[\frac{pmol}{\mu l}\right]=\frac{GFP\left[\frac{ng}{\mu l}\right]\times 10^3}{size\times 650}=\frac{35\left[\frac{ng}{\mu l}\right]\times 10^3}{765\times 650}=0.07\left[\frac{pmol}{\mu l}\right]$$

$$1C3st.4\left[\frac{pmol}{\mu l}\right]=\frac{1C3st.4\left[\frac{ng}{\mu l}\right]\times 10^3}{size\times 650}=\frac{94\left[\frac{ng}{\mu l}\right]\times 10^3}{2300\times 650}=0.063\left[\frac{pmol}{\mu l}\right]$$

1C3:plux-ter st.4 was diluted x2
Reaction mix:

component	Volume[µl]	In?
Gibson assembly master mix(x2)	15	+
1C3:Plux-ter st.4 (lin) (0.05pmol)	1.6	+
GFP (lin) (0.15 pmol)	2.2	+
UPW	1.2	+
tot	20	V

Incubation at 50 ^{0}C for 1 hr.at 15:55

Store in -20 ^{0}C

Part name	Gib st.4
date	16/06/2014
-20 ^{0}C storage	Gate3+M13\H8

Transformation to top 10

20 min frost on ice 100 µL
5 µL of 1C3:Plux-GFP (st.4)
30 min on ice
1 min heat shock 42 ^{0}C
2 min on ice

incubation overnight.

Colony PCR

13/06/2014

Primer name	**forward/ reversed**	**Tm**	**Add DMSO?**
pLux f st.2	**forward**	69	yes
ter r st.2	**reversed**	69	yes

Reaction mix: make total mix and then divide 20 [µl] to each of 7 PCR tube

component	Volume[µl] for 1 colony	For total mix	In?
Taq ready mix (x2)	10	70	
DMSO	0.5	3.5	
pLux f st.2 (10 ng/µl)	1	7	
ter r st.2 (10 ng/µl)	1	7	
1C3:PLux-GFP	colony	----	
UPW	7.5	52.5	
tot	20	140	

PCR program

stage	Temp C^0	time
Initial denaturation	94	3 min
35 cycles	94	30 sec
	68	30 sec
	72	1 min
Final extension	72	10 min
hold	4	

Run gel agarose 1% for product validation

Positive : 1000 bp
negative : 270 bp

Strategy Failed!

second strategy

Preparation of the G-blocks

7.7.14

Preparation of the G-blocks

Protocol

1. Centrifuge the tube from IDT in order to get all of the DNA at the bottom.
2. Add UPW to get DNA concentration of 20 ng/μL (initial amount of DNA in the tube is 200ng).
3. Mix by vortex.
4. Store the stock tube in the -20°C freezer.

Storage

Sample name	Box	Location
GB1 (original tube from IDT)	Gate 3- Alex and Noa	A1
GB2 (original tube from IDT)	Gate 3- Alex and Noa	A2

Amplification of pSB1C3

15.7.14

Amplification of pSB1C3

We wanted to get sufficient amount of the plasmid, so we decided to amplify the plasmid with PCR.

Reaction tube composition:

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 f [10μM]	2.5
Reverse primer: 1C3 r [10μM]	2.5
Template: pSB1C3 [2 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

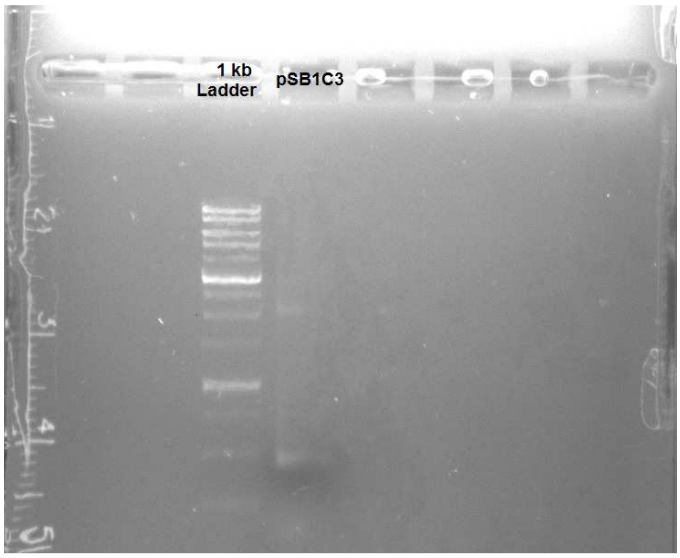
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	66	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: 2070 bp.

After the PCR reaction we ran the product in 1% agarose gel.

The gel image



We can see that the 2070 bend is really weak.

In order to purify the DNA we did gel extraction.
The product concentration after the clean up was 12.2 ng/μl.

Gibson assembly (first try) and Transformation (first try)

16.7.14

Gibson assembly (first try)

In order to insert the G-blocks into the pSB1C3 plasmid we did a Gibson assembly reaction.

Transformation (first try)

We did a transformation of the Gibson product to TOP10 bacteria. We plated the bacteria on LB+CM plates (100 μL and rest).

Colony PCR and starters

17.7.14

Transformation results

After an overnight incubation we saw colonies on both plates.

Colony PCR

In order to verify that the G-blocks were inserted into the plasmid we did a colony PCR.
6 colonies were taken for the PCR.

Reaction tube composition:

Component	Volume [μl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10μM]	2
Reverse primer: pSB1C3 ver AS [10μM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

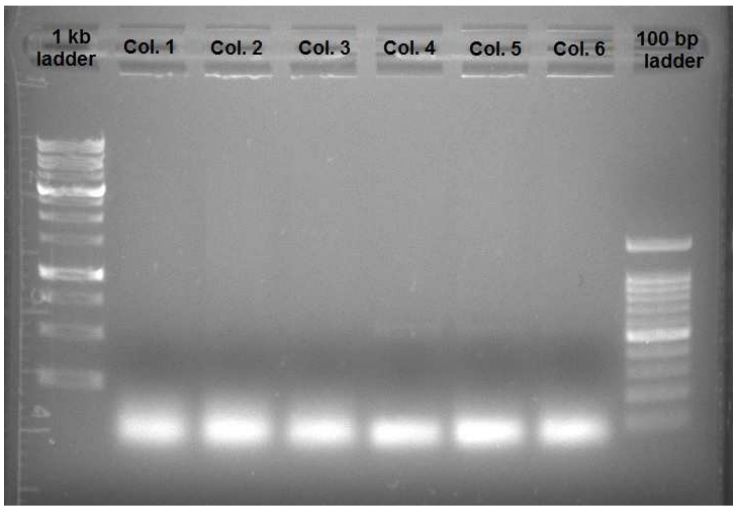
- The primers for the PCR are the pSB1C3 sequencing primers.

- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

- pSB1C3 Ver AS:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp

The gel image



From the gel we saw that the colony PCR wasn't positive nor negative for the desired clone.

Starters

Because we didn't get any result, positive nor negative, in the colony PCR we decided to try a restriction enzymes protocol in order to check if the G-blocks got into the pSB1C3 plasmid.
We made 10 starters from 10 colonies that grew on the rest plate so that the next day we will have 10 mini-preps to use in the restriction protocol. We also made a back-up plate for the 10 colonies.

Enzymes digestion (first try)

18.7.14

Enzymes digestion (first try)

In the morning we saw that colony 6 didn't grow on the back-up plate so we didn't use that starter.
We did a mini-prep for 9 starters.

DNA concentrations after the mini-prep

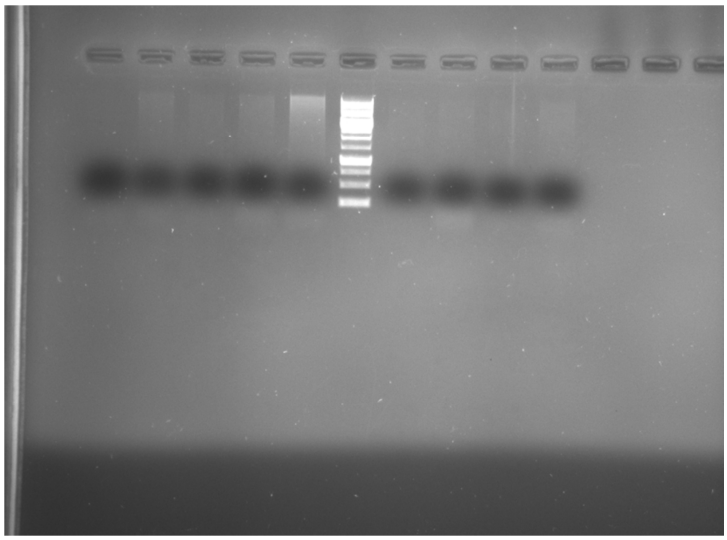
Colony number	DNA concentration
1	29
2	45.5
3	38
4	49.5
5	50
7	60.5
8	35.5
9	54
10	56

Reaction tube composition:

Colony number	Enzyme- PstI (µl)	Buffer cut smart (µl)	MBW (µl)	DNA from mini-prep (µl)	Total (µl)
1	0.5	2	0.5	17	20
2	0.5	2	7.5	10	20
3	0.5	2	5	12.5	20
4	0.5	2	7.5	10	20
5	0.5	2	7.5	10	20
7	0.5	2	9.5	8	20
8	0.5	2	4.5	13	20
9	0.5	2	8.5	9	20
10	0.5	2	8.5	9	20

If the insert is in the plasmid, an expected band would be around 3600 bp, otherwise, 2070 bp.

The gel image



We didn't see any bend in the gel.

- Because we didn't get any bend in the colony PCR gel and in the Enzyme digestion gel we thought that the colonies that grew on the CM plates after the transformation were contamination.

Starters

19.7.14

Starters

6 colonies were taken from the back-up plate for the starters so that the next day we will have 6 mini-preps to use in the restriction protocol. We also made a back-up plate for the 6 colonies.

Enzymes digestion (second try) and Transformation (second try)

20.7.14

Enzymes digestion (second try)

We did another attempt to cut with the same restriction enzyme as for the last attempt in order to see if the insert got into the plasmid.

DNA concentrations after the mini-prep

Colony number	DNA concentration
1	57.5
2	97.5
3	102.5
4	101.5
5	88.5
6	78

Reaction tube composition:

Colony number	Enzyme- PstI (µl)	Buffer cut smart (µl)	MBW (µl)	DNA from mini-prep (µl)	Total volume (µl)
1	0.5	2	8.5	9	20
2	0.5	2	12	5.5	20
3	0.5	2	12.5	5	20
4	0.5	2	12.5	5	20
5	0.5	2	11.5	6	20
6	0.5	2	10.5	7	20

If the insert is in the plasmid, an expected band would be around 3600 bp, otherwise, 2070 bp.

The gel image

In the gel we saw that all the colonies were negative again.

Transformation (second try)

In order to check if the antibiotic was the problem for the contamination we saw in the colony PCR results and in the enzymes digestion results, we made new LB plates with new CM.
Then we tried to do a new transformation of the Gibson product (from 16.7.14) to TOP10 bacteria.

Storage

Sample name	Box	Location
CM	Gate 3- Alex and Noa	A3

Amplification of pSB1C3, Gibson assembly (second try) and Transformation (third try)

21.7.14

Transformation results

Nothing grow on the plates, so we figured that the colonies that grow in the first transformation were contamination .

Amplification of pSB1C3 from 2014 distribution kit

We decided to try a new Gibson reaction, with the new pSB1C3 from the 2014 distribution kit.
First, a PCR was done to amplify the plasmid .

Reaction tube composition:

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 f [10μM]	2.5
Reverse primer: 1C3 r [10μM]	2.5
Template: pSB1C3 [1 ng/μl]	10
Phusion hot start	0.5
DMSO	1.5
UPW	22
Total	50

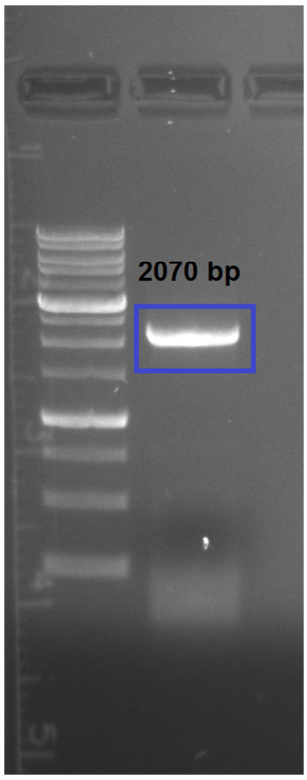
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	70	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: 2070 bp.

The gel image

- 1 kb ladder on the left.



Concentration

pSB1C3- 57.5 ng/µl.

Storage

Sample name	Box	Location
pSB1C3 21.7.14	Gate 3- Alex and Noa	A4

Gibson assembly (second try)

We did a new Gibson assembly reaction with the plasmid pSB1C3 from the 2014 distribution kit [57.5 ng/µl].

Transformation (third try)

We did a transformation of the Gibson assembly product to TOP10 bacteria. We plated the bacteria on LB+CM plates (100 µL and rest)

Colony PCR

22.7.14

Transformation results

After an overnight incubation we saw colonies on both plates.

Colony PCR

In order to verify that the G-blocks were inserted into the plasmid we did a colony PCR.
6 colonies were taken for the PCR.

Reaction tube composition:

Component	Volume [μl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10μM]	2
Reverse primer: pSB1C3 ver AS [10μM]	2
Template	1 colony
UPW	6
Total	20

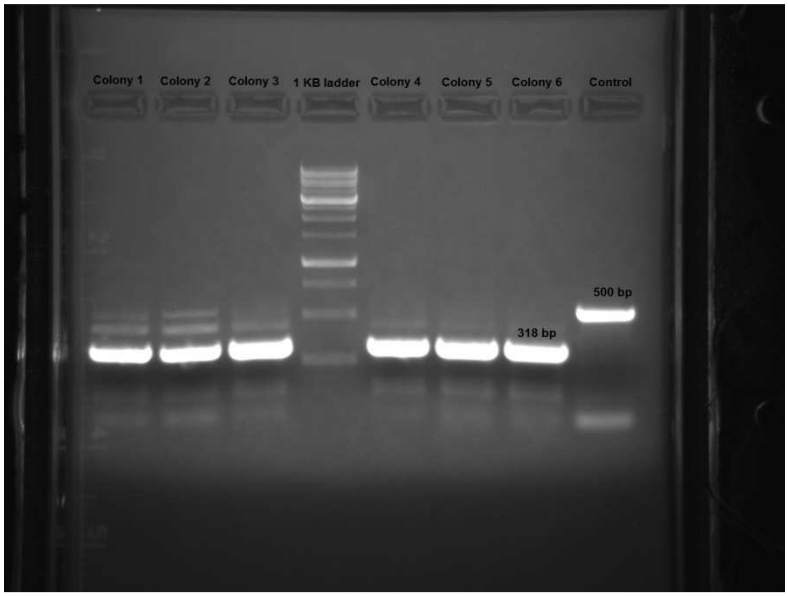
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

- The primers for the PCR are the pSB1C3 sequencing primers.
- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.
- pSB1C3 Ver AS:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp

The gel image



From the image we can see that the colonies were negative for the desired clone. We can also see that there are unspecific products in the gel and that there was probably a contamination in the tubes (the 500 bp band).

10 colonies were taken for a new colony PCR and we also did a control tube without a colony. We have used new primers and new UPW for the reaction.

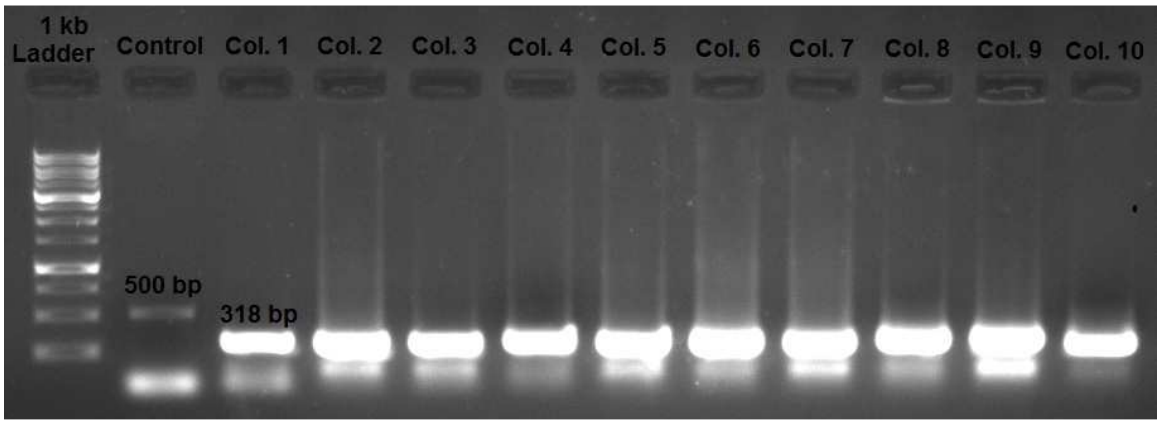
Reaction tube composition:

Component	**Volume [μl]**
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10μM]	2
Reverse primer: pSB1C3 ver AS [10μM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

The gel image



From the gel we saw that All the colonies were negative for the desired clone.

New strategy for the gate assembly

23.7.14

New strategy for the gate assembly

After our attempts to insert the G-blocks into the pSB1C3 plasmid failed we decided to order new primers for the amplification of the G-blocks and the plasmid.
With the new primers the length of the parts will be:

Part name	Original length (bp)	Length with the new primers (bp)
pSB1C3	2070	2054
GB1	788	805
GB2	811	811

Amplification of the G-blocks

27.7.14

Amplification of GB1

Storage

Sample name	**Box**	**Location**
GB1 [2 ng/µl]	Gate 3- Alex and Noa	A5
GB1 F [10µM]	Gate 3- Alex and Noa	A7
GB1 R [10µM]	Gate 3- Alex and Noa	A8

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: GB1 F [10μM]	2.5
Reverse primer: GB1 R [10μM]	2.5
Template: GB1 [2 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	68	30 sec	35
Extension	72	30 sec	35
Final extension	72	10 min	-
Hold	4	-	-

Amplification of GB2

Storage

Sample name	Box	Location
GB2 [2 ng/μl]	Gate 3- Alex and Noa	A6
GB2 F [10mM]	Gate 3- Alex and Noa	A9
GB2 R [10mM]	Gate 3- Alex and Noa	B1

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: GB2 F [10μM]	2.5
Reverse primer: GB2 R [10μM]	2.5
Template: GB2 [2 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

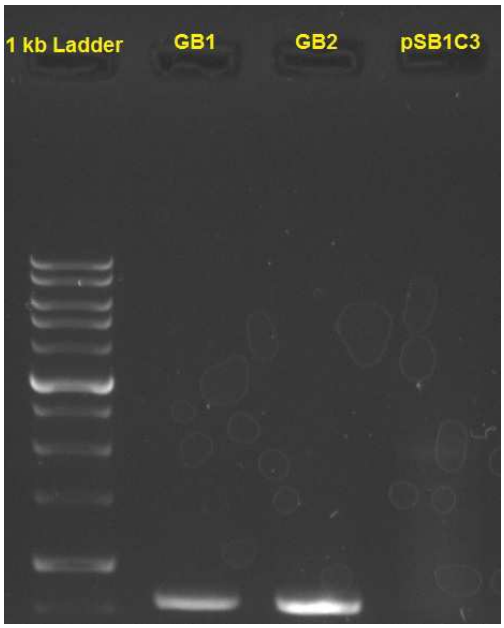
stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	68	30 sec	35
Extension	72	30 sec	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product:

- GB1- 805 bp.
- GB2- 811 bp.

After the PCR reaction we ran the products in 1% agarose gel.

The gel image



From the gel we saw that the PCR reactions for the G-blocks were successful.

DNA concentration after PCR clean-up:

- GB1- 46 ng/µl
- GB2- 53.5 ng/µl

Storage

Sample name	Box	Location
GB1 28.7.14 [46 ng/µl]	Gate 3- Alex and Noa	B2
GB2 28.7.14 [53.5 ng/µl]	Gate 3- Alex and Noa	B3

Amplification of pSB1C3 (first try)

27.7.14

Amplification of pSB1C3 (first try)

In order to amplify the plasmid with the new primers we have designed, we planned a new PCR program.

Storage

Sample name	Box	Location
New pSB1C3 [1 ng/μl]	Gate 3- Alex and Noa	-
1C3 F [10μM]	Gate 3- Alex and Noa	B4
1C3 r GB g3 new overlap [10μM]	Gate 3- Alex and Noa	B5

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 F [10μM]	2.5
Reverse primer: 1C3 r GB g3 new overlap [10μM]	2.5
Template: New pSB1C3 [1 ng/μl]	10
Phusion hot start	0.5
DMSO	1.5
UPW	22
Total	50

PCR program

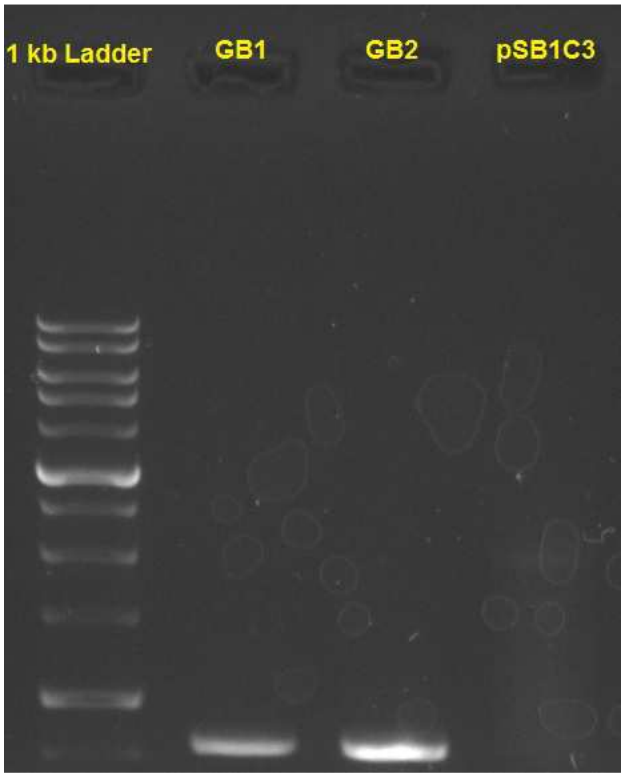
stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	61	30 sec	35

Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: 2054 bp.

After the PCR reaction we ran the product in 1% agarose gel.

The gel image



From the gel image we saw that the PCR reaction for pSB1C3 didn't work.

Amplification of pSB1C3 (second try)

28.7.14

Amplification of pSB1C3 (second try)

After the pSB1C3 PCR didn't work yesterday, we decided to try gradient PCR for the amplification.

Storage

Sample name	Box	Location
pSB1C3 28.7.14 [1 ng/μl]	Gate 3- Alex and Noa	B6
1C3 F [10μM]	Gate 3- Alex and Noa	B4
1C3 r GB g3 new overlap [10μM]	Gate 3- Alex and Noa	B5

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	5
dNTP's [10 mM]	0.5
Forward primer: 1C3 F [10μM]	1.25
Reverse primer: 1C3 r GB g3 new overlap [10μM]	1.25
Template: pSB1C3 28.7.14 [1 ng/μl]	5
Phusion hot start	0.25
DMSO	0.75
UPW	11
Total	25

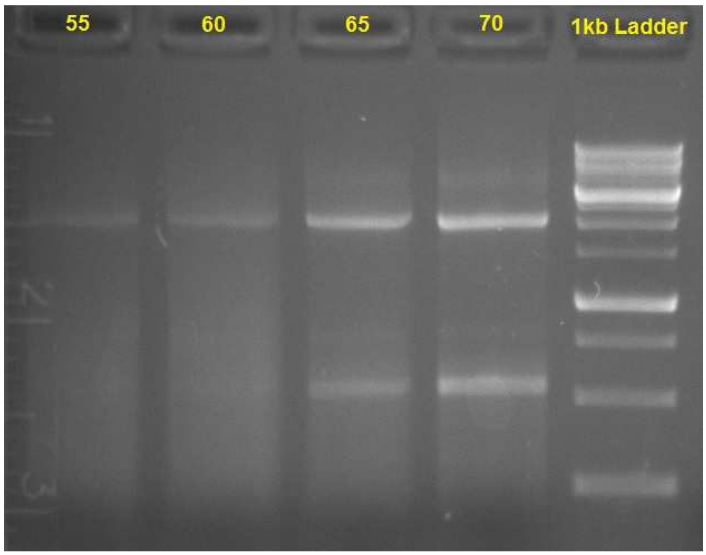
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	55, 60, 65, 70	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: 2054 bp.

After the PCR reaction we ran the product in 1% agarose gel.

The gel image



From the image we saw that the PCR worked, so we took all 4 reaction tubes, combine them together and did a PCR clean-up.
The concentration of the product was 71.6 ng/µl.

Storage

Sample name	Box	Location
pSB1C3 for Gibson [71.6 ng/µl]	Gate 3- Alex and Noa	B7

Gibson assembly and Transformation

29.7.14

Gibson assembly

We did a Gibson assembly in order to insert the G-blocks into the plasmid.

Transformation

We did a transformation of the Gibson product to TOP10 bacteria. We plated the bacteria on LB+CM plates (100 µL and rest).

Colony PCR, G-blocks PCR and pSB1C3 PCR

30.7.14

Transformation results

The transformation to Top10 bacteria worked- we saw colonies on both plates- 100µl and rest.

Colony PCR

We did a colony PCR to verify the insertion of the G-blocks into the plasmid. Overall we checked 16 colonies in the colony PCR.

Reaction tube composition

Component	Volume [µl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10µM]	2
Reverse primer: pSB1C3 ver AS [10µM]	2
Template	1 colony
UPW	6
Total	20

PCR program

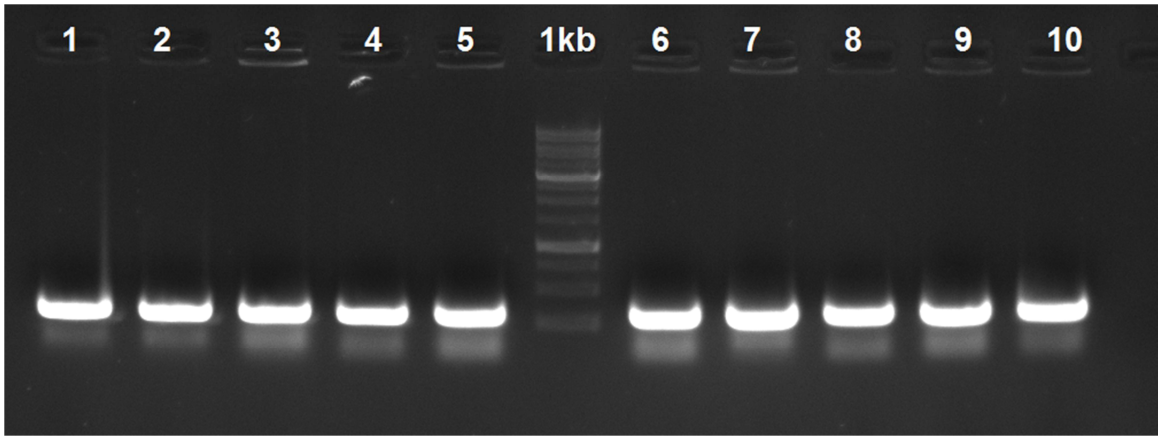
stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

- The primers for the PCR are the pSB1C3 sequencing primers.
- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

pSB1C3_Ver_AS:
CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
Tm: 56.7°C.

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp

The gel image



The colony PCR showed negative result for every colony- the bands were about 300 bp. We figured that the plasmid probably was self-ligated.

Therefore, we decided to do a PCR to the Gibson assembly product so see if the two G- blocks had been ligated. We also tried to amplify the pSB1C3 plasmid so we will have enough sample for the future.

Amplification of pSB1C3 and the Gibson product

For the plasmid

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: 1C3 F [10μM]	2.5
Reverse primer: 1C3 r GB g3 new overlap [10μM]	2.5
Template: pSB1C3 for Gibson [2 ng/μl]	5
Phusion hot start	0.5
DMSO	1.5
UPW	27
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	60	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

For the Gibson product

Reaction tube composition

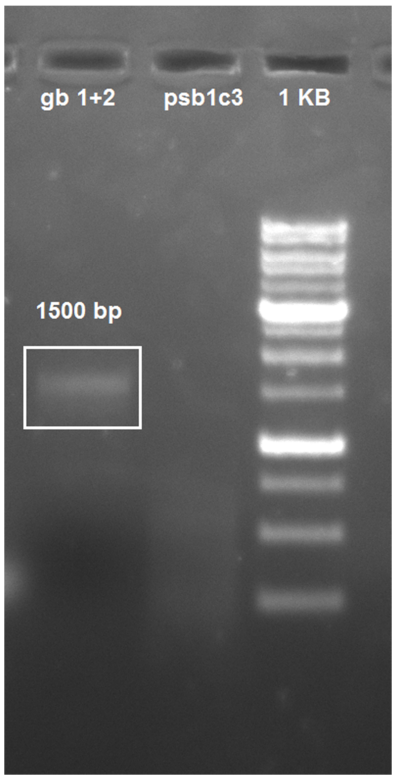
Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1

Forward primer: GB1 F [10μM]	2.5
Reverse primer: GB2 R [10μM]	2.5
Template: Gibson product	2
Phusion hot start	0.5
DMSO	1.5
UPW	30
Total	50

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	68	30 sec	35
Extension	72	1 min	35
Final extension	72	10 min	-
Hold	4	-	-

The gel image



From the image we saw that the G-blocks were ligated in the Gibson assembly reaction and that the amplification of pSB1C3 didn't work.

Enzymes digestion, ligation and transformation

31.7.14

Enzymes digestion

Instead of inserting the G-blocks into the plasmid with Gibson assembly we decided to cut both the plasmid and the clean Gibson product (GB1+GB2) with restriction enzymes- EcoRI and PstI, and then to do a ligation reaction.

For the Gibson product

Reaction tube composition

Component	Volume [µl]
Clean DNA	30
PstI enzyme (HF)	1
EcoRI enzyme (HF)	1
CutSmart buffer [X10]	3.5
Total	35.5

For the plasmid

Reaction tube composition

Component	Volume [µl]
Clean DNA [71.6 ng/µl]	20
PstI enzyme (HF)	1
EcoRI enzyme (HF)	1
CutSmart buffer [X10]	2.5
Total	24.5

Incubation

The reaction tubes were in 37°C for 45 minutes.

Clean-up

After the restriction we did a PCR clean-up to both tubes and the concentrations we got:
GB1+GB2- 8.9 ng/µl.
pSB1C3 after restriction- 25.5 ng/µl.

Ligation

In order to ligate the G-blocks to the plasmid we did a ligation reaction as follows:

Reaction tube composition

Component	Volume [µl]
Insert	12.6
Vector	2
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	2.4
Total	20

Incubation

The reaction tubes were in 30°C for 1 hour.

Storage

Sample name	Box	Location
1C3+GB1+GB2 ligation 31.7.14	Gate 3- Alex and Noa	B8

Transformation

5 µL from the ligation reaction were transformed to TOP10 bacteria.

Transformation results

1.8.14

Transformation results

The transformation to Top10 bacteria worked- we saw colonies on both plates- 100µl and rest.

Colony PCR and starters for pSB1C3

3.8.14

Colony PCR

We did a colony PCR to verify the insertion of the G-blocks into the plasmid. Overall we checked 6 colonies in the colony PCR.

Reaction tube composition

Component	Volume [µl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10µM]	2
Reverse primer: pSB1C3 ver AS [10µM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

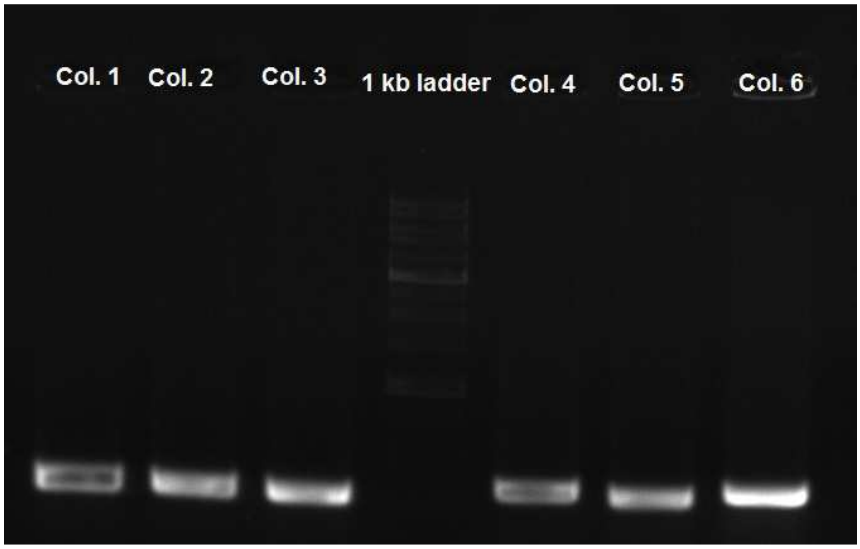
- The primers for the PCR are the pSB1C3 sequencing primers.

- pSB1C3 ver S:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

- pSB1C3 Ver AS:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp.

The gel image



Again we saw that the plasmid was self-ligated.

Starters

Because the plasmid was self-ligated in our previous attempts to insert the G-blocks into the plasmid, we decided to try to do a CIP reaction to the plasmid after it was digested with EcoRI and PstI.

In order to have enough amount of the circular plasmid for the digestion, we made 4 starters from the glycerol stock of the 1C3 plasmid.

Mini prep, enzymes digestion and CIP

4.8.14

Mini-prep for pSB1C3

We did a mini-prep for each starter from yesterday.

Concentrations and storage

Sample name	Box	Location
pSB1C3 after m.p [156.5 ng/µl]	-	-
pSB1C3 after m.p [124.7 ng/µl]	Gate 3- Alex and Noa	B9
pSB1C3 after m.p [129.7 ng/µl]	Gate 3- Alex and Noa	C1
pSB1C3 after m.p [167.2 ng/µl]	Gate 3- Alex and Noa	C2

Enzymes digestion

In order to ligate the plasmid and the G-blocks we cut the plasmid with EcoRI and PstI.
For the reaction we Took 3000 ng from the clean plasmid.

Reaction tube composition

Component	Volume [µl]
pSB1C3 after m.p [167.2 ng/µl]	17.9
PstI enzyme (HF)	1.5
EcoRI enzyme (HF)	1.5
Cut smart buffer	3
MBW	6.1
Total	30

Incubation

The reaction tube was in 37°C for 30 minutes.

Clean-up

After the incubation we cleaned the digested product.

CIP reaction

In order get the plasmid pSB1C3 dephosphorylated we did a CIP reaction.

Reaction tube composition

Component	Volume [µl]
Clean DNA after restriction	30
Buffer 3 [X10]	3.4
CIP	1
Total	34.4

Incubation

The reaction tube was in 37°C for 4 hours.

Clean-up

After the incubation we cleaned the product. The concentration of the clean product was 12.3 ng/µl.

Storage

Sample name	Box	Location
pSB1C3 after CIP [12.3 ng/µl]	Gate 3- Alex and Noa	C3

Gibson assembly, amplification of the product, enzymes digestion and ligation

6.8.14

Gibson assembly

In order to ligate GB1 to GB2 we did a Gibson reaction.

Reaction tube composition

Component	Volume [µl]
GB1 [46 ng/µl]	1.1
GB2 [53.5 ng/µl]	1
UPW	2.9
Gibson mix	15
Total	20

Incubation

The reaction tube was in 50°C for 60 minutes.

Storage

Sample name	Box	Location
GB1+GB2 Gib	Gate 3- Alex and Noa	C4

Amplification of the Gibson product

In order to have enough amount of the Gibson product we did PCR reaction.

Reaction tube composition

Component	Volume [μl]
phusion reaction buffer [x5]	10
dNTP's [10 mM]	1
Forward primer: GB1 F [10μM]	2.5
Reverse primer: GB2 R [10μM]	2.5
Template: Gibson product	1/2
Phusion hot start	0.5
DMSO	1.5
UPW	31/30
Total	50

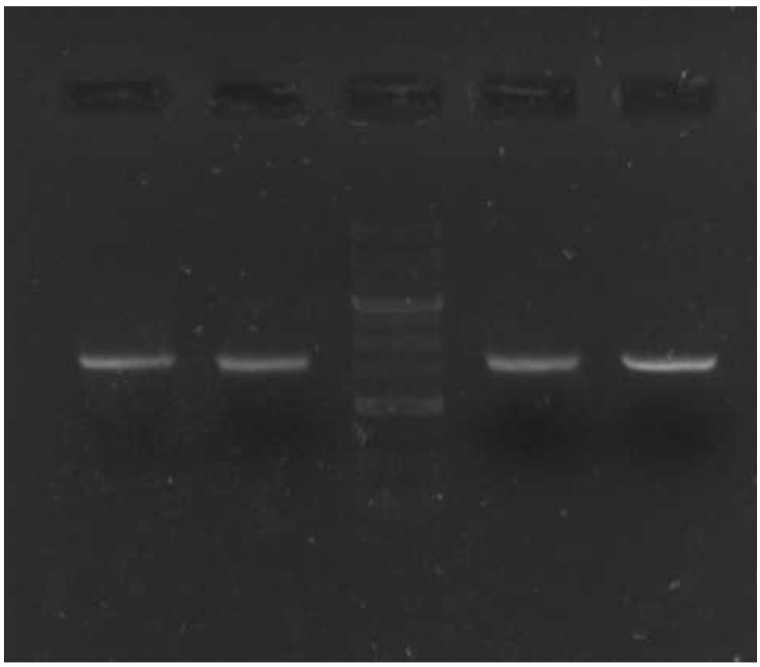
PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	98	30 sec	-
Denaturation	98	10 sec	35
Annealing	70	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

The expected length of the PCR product: 1534 bp.

After the PCR reaction we ran the products in 1% agarose gel.

The gel image



The samples we ran from left to right:

- 1 μL of Gibson product, first duplicate.
- 1 μL of Gibson product, second duplicate.
- 1 kb ladder.
- 2 μL of Gibson product, first duplicate.
- 2 μL of Gibson product, second duplicate.

From the gel we saw that the PCR reactions for the Gibson product were successful and we got a band around 1500 bp for the reactions.

Clean-up

We took all 4 reaction tubes, combine them together and did a PCR clean-up.
The concentration of the product was 267 ng/μl.

Enzymes digestion

In order to ligate the plasmid and the G-blocks we cut the Gibson product with EcoRI and PstI.
For the reaction we Took all the DNA after clean-up.

Reaction tube composition

Component	Volume [μl]
Gibson product [267 ng/μl]	30.5
PstI enzyme (HF)	1.5
EcoRI enzyme (HF)	1.5
Cut smart buffer	4
MBW	2.5
Total	40

Incubation

The reaction tube was in 37°C for 30 minutes.

Clean-up

We did a PCR clean-up to get clean digested product.
The concentration after clean-up was 171.3 ng/μl.

Storage

Sample name	Box	Location
GB1+GB2 after restriction enzymes 6.8.14	Gate 3- Alex and Noa	C5

Ligation

In order to ligate the Gibson product to the plasmid we did 3 ligation reactions: ratio of 1:3 (vector to insert), ratio of 1:5 and no insert.

Reaction tube composition- for the 1:3 ratio

Component	Volume [μl]
Insert	0.7
Vector	4.1
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	12.2
Total	20

Reaction tube composition- for the 1:5 ratio

Component	Volume [µl]
Insert	1.1
Vector	4.1
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	11.8
Total	20

Reaction tube composition- for the no insert

Component	Volume [µl]
Insert	-
Vector	4.1
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	12.9
Total	20

Incubation

The reaction tubes were in 16°C overnight.

Storage

Sample name	Box	Location
pSB1C3- gate 3, no insert 6.8.14	Gate 3- Alex and Noa	C6
pSB1C3- gate 3, 1:3 6.8.14	Gate 3- Alex and Noa	C7

Transformation

7.8.14

Transformation

5 µL from each ligation reaction (1:3, 1:5 and no insert) were transformed to TOP10 bacteria.

Transformation results and colony PCR

8.8.14

Transformation results

The transformation to Top10 bacteria worked- we saw colonies on every plate. We noticed that the same amount of colonies grow on the no insert plates as for the 1:3 and the 1:5 plates. We expected to get far less colonies on the no insert plates, because if the plasmid is after CIP reaction and there is no insert in the reaction tube, it doesn't supposed to get self ligated. We decided to try colony PCR to check if we can find colonies with the insert.

Colony PCR

We did a colony PCR to verify the insertion of the G-blocks into the plasmid. Overall we checked 6 colonies in the colony PCR.

Reaction tube composition

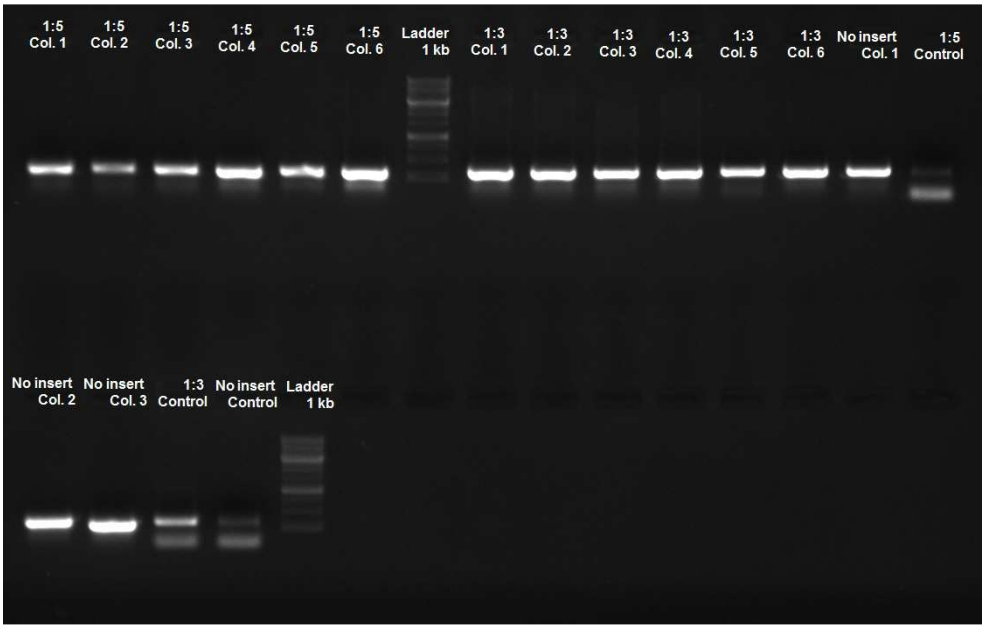
Component	Volume [µl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10µM]	2
Reverse primer: pSB1C3 ver AS [10µM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp.

The gel image



The control tubes didn't contain colony.

The colony PCR showed negative result for every colony- the bands were about 300 bp.

Enzymes test and enzymes digestion

10.8.14

Enzymes test

In order to check if our enzymes are the cause for the pSB1C3 self ligation (the enzymes don't cut as expected), we decided to run three reaction as follows:

First reaction:

Reaction tube composition

Component	Volume [μl]
pSB1C3 after m.p (167.2 ng/μl)	3
Enzyme PstI HF	0.5
CutSmart Buffer [X10]	2
MBW	14.5
Total	20

Incubation

The reaction tube was in 37°C for 30 minutes.

Second reaction:

Reaction tube composition

Component	Volume [μl]
pSB1C3 after m.p (167.2 ng/μl)	3
Enzyme EcoRI HF	0.5
CutSmart Buffer [X10]	2
MBW	14.5
Total	20

Incubation

The reaction tube was in 37°C for 30 minutes.

Third reaction:

Reaction tube composition

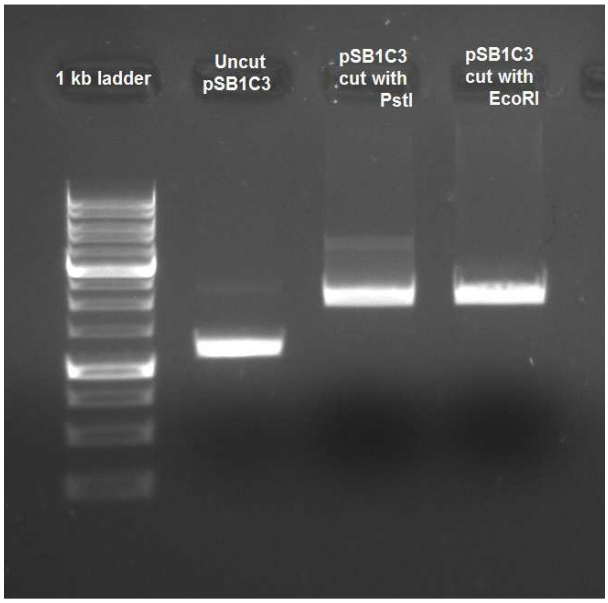
Component	Volume [μl]
pSB1C3 after m.p (167.2 ng/μl)	3
CutSmart Buffer [X10]	2
Total	5

Incubation

No incubation.

After the incubation we ran the samples in 1% agarose gel.

The gel image



From the gel we saw that with the enzyme EcoRI we got 2070 bp band as expected, but for the restriction with PstI we got another band except for the 2070 bp band- about 4000 bp.

We decided to try a ligation protocol again for the plasmid and the Gibson product (GB1+GB2), so we cut the plasmid again with new enzymes.

Enzymes digestion

Reaction tube composition

Component	Volume [µl]
pSB1C3 after m.p [156.5 ng/µl]	31
PstI enzyme	2
EcoRI enzyme	2
Buffer 3.1 [X10]	3.9
Total	38.9

Incubation

The reaction tube was in 37°C for 2 hours.

Clean-up
After the incubation we cleaned the product with the PCR clean-up kit.

CIP, ligation and transformation

11.8.14

CIP reaction

In order get the plasmid pSB1C3 dephosphorylated we did a CIP reaction. This time we used a different CIP.

Reaction tube composition

Component	Volume [µl]
Clean DNA after restriction	32
Buffer 3 [X10]	3.6
CIP	1
Total	36.6

Incubation

The reaction tube was in 37°C for 1 hours.

Clean-up

After the incubation we cleaned the product. The concentration of the clean product was 14.3 ng/µl.

Ligation

In order to ligate the Gibson product to the plasmid we did 3 ligation reactions: ratio of 1:3 (vector to insert), ratio of 1:5 and no insert.

Reaction tube composition- for the 1:3 ratio

Component	Volume [µl]
Insert [171.3 ng/µl]	0.7
Vector [14.3 ng/µl]	3.5
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	12.8
Total	20

Reaction tube composition- for the 1:5 ratio

Component	Volume [µl]
Insert [171.3 ng/µl]	1.1
Vector [14.3 ng/µl]	3.5
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	12.4
Total	20

Reaction tube composition- for the no insert

Component	Volume [µl]
Insert [171.3 ng/µl]	-
Vector [14.3 ng/µl]	3.5
10X T4 DNA Ligase Buffer	2
T4 DNA Ligase	1
MBW	13.5
Total	20

Incubation

The reaction tubes were in 30°C for 2 hours.

Transformation

5 µL from each ligation reaction (1:3, 1:5 and no insert) were transformed to TOP10 bacteria.

Transformation results and plate reader preparations

12.8.14

Transformation results

The transformation to Top10 bacteria worked- we saw colonies on every plate. Again we noticed that the same amount of colonies grow on the no insert plates as for the 1:3 and the 1:5 plates.
Because of it we decided not to do colony PCR this time, but to try and check if there are colonies with high GFP emission.

Plate reader preparations

In order to check if any of the colonies have high GFP emission, we needed to grow starters.
We took 4 plates of 48 wells, in every well we put 1.5 ml of LB+CM and one colony from one of the ligation plates. We grew the bacteria over night in a 37°C shaker.

Plate reader measurement, colony PCR and enzymes digestion

13.8.14

Plate reader measurement

We transferred 200 μL from every well to 96 wells plate and then we measured the GFP emission of every well. We found 8 wells with high GFP emission.

Colony PCR

We did a colony PCR to check if our 8 colonies had the insert with the GFP. We also made a back-up plate with the colonies.

Reaction tube composition

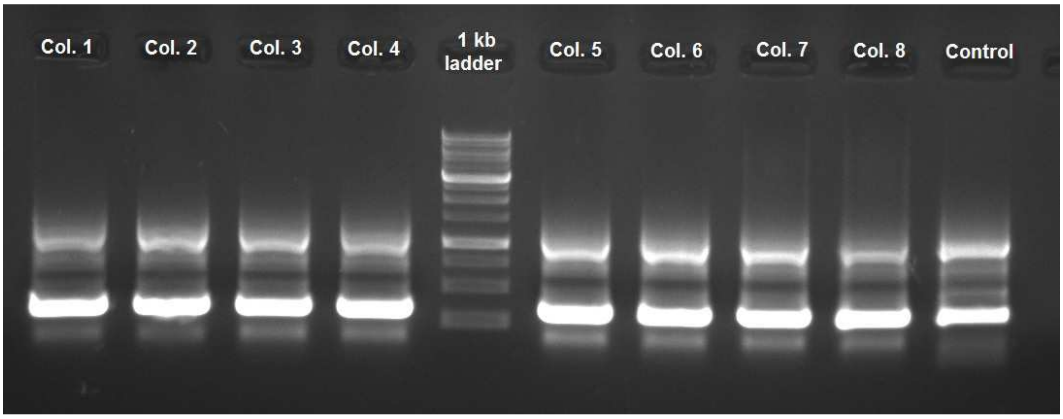
Component	Volume [μl]
Taq ready mix [x2]	10
Forward primer: pSB1C3 ver S [10μM]	2
Reverse primer: pSB1C3 ver AS [10μM]	2
Template	1 colony
UPW	6
Total	20

PCR program

stage	Temp (°C)	Time	Number of cycles
Initial denaturation	94	3 min	-
Denaturation	94	30 sec	35
Annealing	55	30 sec	35
Extension	72	1:45 min	35
Final extension	72	10 min	-
Hold	4	-	-

The Insert length is 1534 bp.
Positive result: total DNA length expected in gel, plasmid with insert- 117+155+1534+23+23=1852 bp.
Negative result: Total DNA length expected in gel, plasmid without insert- 272+46=318 bp.

The gel image



The control tube didn't contain colony.

The colony PCR showed negative result (300 bp) for every colony and also a band of 750 bp.

Enzymes digestion

Because we wanted to double check our 8 colonies we decided to check them with restriction enzymes. We did 8 mini-preps, one for each colony, for the restriction.

DNA concentration after mini-prep

Colony number	Concentration (ng/μl)
1	40.3
2	59.5
3	49.9
4	45.3
5	40.4
6	45.9
7	41.7
8	46.9

After the mini-prep we made 8 reaction tubes for the restriction.

Reaction tube composition- for colony 2

Component	Volume [µl]
DNA after m.p [59.5 ng/µl]	15
PstI HF enzyme	0.5
EcoRI HF enzyme	0.5
MBW	6.5
Buffer CutSmart [X10]	2.5
Total	25

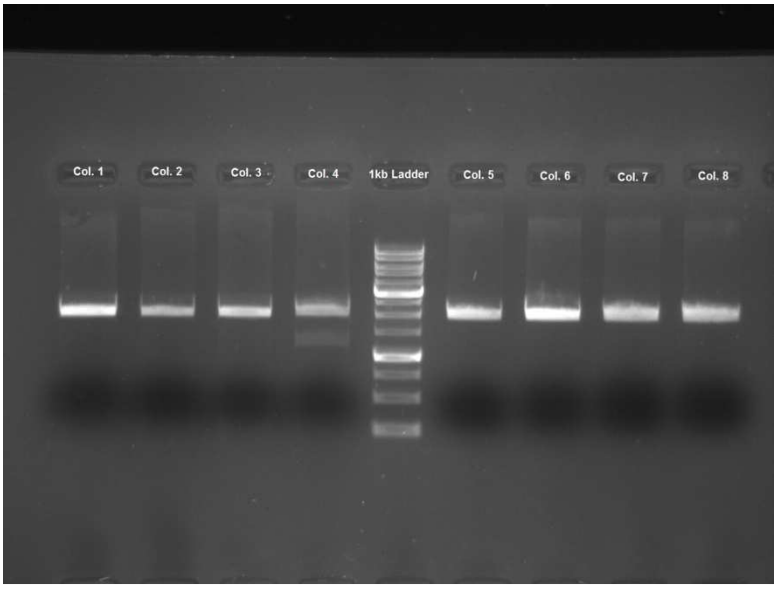
Reaction tube composition- for the other colonies

Component	Volume [µl]
DNA after m.p [59.5 ng/µl]	20
PstI HF enzyme	0.5
EcoRI HF enzyme	0.5
MBW	1.5
Buffer CutSmart [X10]	2.5
Total	25

Incubation

The reaction tubes were in 37°C for 30 minutes.

The gel image



For colony 4 we saw the 1500 bp band, the length of the insert. We decided to send a sample of colony 4 to sequencing to verify that the plasmid contains the insert.

Sequencing

14.8.14

Sequencing of pSB1C3-pLux-GFP-LuxI

We sent the plasmid to sequencing with the primers:

- pSB1C3_seq_F:
  TGCCACCTGACGTCTAAGAAACC (located 117 bp from the beginning of the insert).
  Tm: 57°C.

- pSB1C3_seq_R:
  CCGTATTACCGCCTTTGAGTGAG (located 155 bp from the ending of the insert).
  Tm: 56.7°C.

Sequencing results

18.8.14

Sequencing results

The reaction didn't succeed because the enzyme fell in both tries.

▲ # ▼	▲ File Name▼	▲ Load Date▼
180741	Aug21_33_130897_pSB1C3-GFP-luxI-17-8-14_1C3-ver-S-17-8.ab1	19/08/2014 11:11:01
180742	Aug21_33_130897_pSB1C3-GFP-luxI-17-8-14_1C3-ver-S-17-8.seq	19/08/2014 11:11:01
181055	Aug25_04_130897_pSB1C3-GFP-luxI-17-8-14_1C3-ver-S-17-8.ab1	21/08/2014 09:45:40
181056	Aug25_04_130897_pSB1C3-GFP-luxI-17-8-14_1C3-ver-S-17-8.seq	21/08/2014 09:45:40

Status: Done

Remark:

```
The enzyme fall from reaction
 I will repeat with DMSO

the same
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